6-1-2007

Investigations of Mechanisms Involved in LPS-Stimulated Osteoclastogenesis

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Investigations of Mechanisms Involved in LPS-Stimulated Osteoclastogenesis

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A Thesis

Submitted in Partial Fulfillment of the

Requirement for the Degree of

Master of Dental Science

at the

University of Connecticut

2007

APPROVAL PAGE

Master of Science Thesis

Investigation of Mechanisms Involved in LPS-Stimulated Osteoclastogenesis

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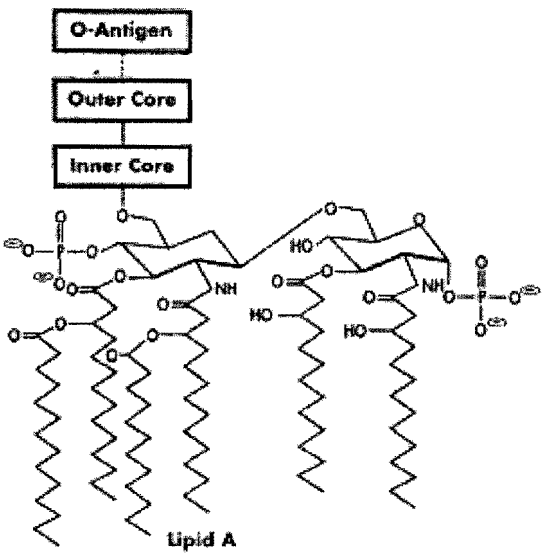
Inflammation is an essential response of the host from infection or tissue invasion and it plays an important role in health and disease. The immune system exerts tight control on the inflammatory response, and most of the time elimination of microorganisms occur with no clinically detectable signs of inflammation (Heumann 2002). However, in certain conditions, such as bacterial infection of the dental pulp , the inflammatory process stimulates localized pathological processes which results initially in pulpal destruction and ultimately in the development of a periradicular lesion accompanied by bone resorption (Stashenko 1994). Gram-negative anaerobes, which contain lipopolysaccharide (LPS or endotoxin) in their cell walls, are the predominant bacteria involved in endodontic infections (Moller 1981). In samples taken from necrotic pulp (Dahlen 1980) and from the dentin wall of periapically involved teeth (Horiba 1990), the presence of LPS has been reported; thereby, establishing its role in periapical bone resorption. It has also been shown that levels of LPS increased with time after periapical lesion induction in rats (Yamasaki 1992) and in other animal models, application of LPS to dental pulp initiated and sustained apical periodontitis (Dahlen 1981).

LPS is the major component of the outermost membrane of Gram-negative bacteria and was the first component of bacteria shown to induce bone resorption (Lynn 1992). It is a complex glycolipid which consists of a variable polysaccharide domain covalently bound to a diglucosamine based acylated phospholipid, lipid A. (Raetz 1990). The lipid A portion of LPS is highly conserved between different strains of Gram-negative bacteria, has been shown to have LPS agonist activity;

therefore, it is thought to be responsible for most of the biological effects of LPS. (Morrison 1987).



Guha et al., Cellular Signalling, 2001

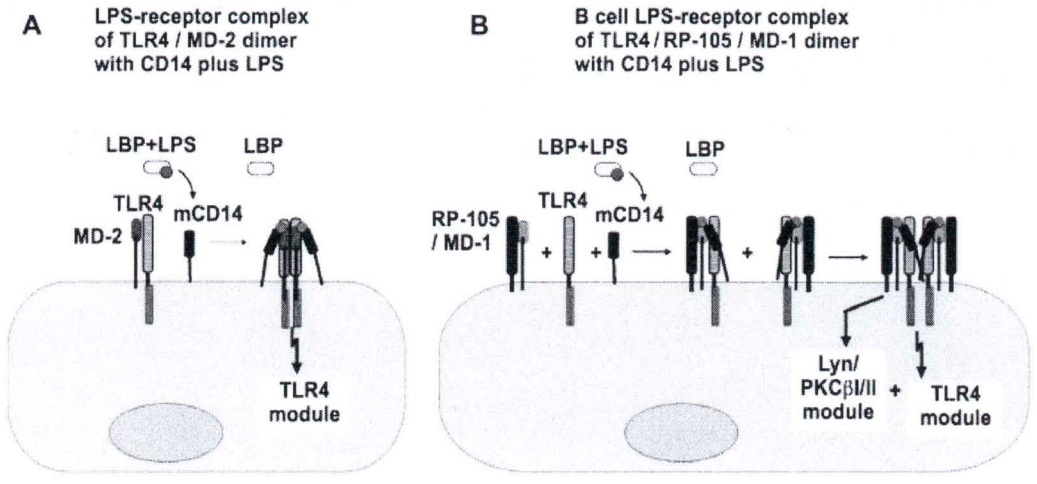
LPS Recogniton

Until 1990, little was known about the protein molecules that specifically recognized LPS and initiated a host response. It was thought that immune cells were activated by LPS through spontaneous intercalation of lipid A into the mammalian lipid bilayer (Lynn 1992). This concept was repudiated with the cloning and sequencing of lipopolysaccharide-binding protein (LBP) by Schumann et al in 1990 (Schumann 1990). LBP is a 60kd serum glycoprotein produced in the liver and catalyzes the transfer of bacterial membrane LPS to its receptor, CD14 from the outer membrane (Schumann 1990). It contains a high affinity binding site for lipid A, opsonizes particles bearing LPS, and mediates macrophage attachment to these particles (Schumann 1990). Normal serum contains LBP at quantities of less than 0.5ug/ml, with a rise to 50ug/ml within twenty-four hours after an acute phase response (Schumann 1990). LBP has been shown to be specifically involved in

recognizing the products of pathogenic bacteria. (Schumann 1996). LPS processing by LBP and CD14 not only initiates a LPS response, it also facilitates clearance of LPS (Miyake 2004). Both LBP and CD14 can cause LPS to lose its biological activity by delivering LPS to high-density lipoprotein (HDL) particles where it is then excreted by the liver (Miyake 2004).

CD14

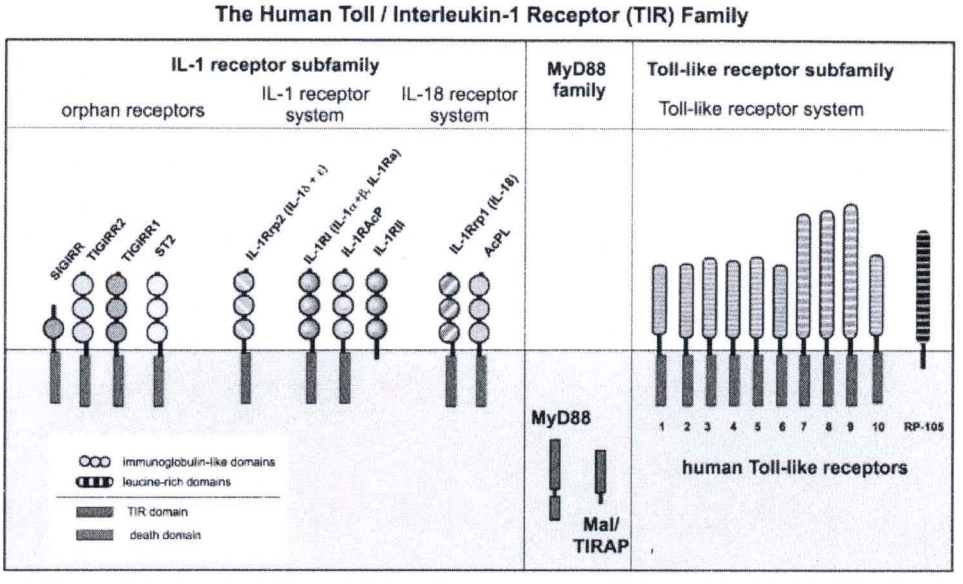
CD14 is an opsonic receptor for complexes of LPS (or LPS-containing particles such as bacteria) and LBP (Aderem 2000). It is either present on the surface of monocytes/macrophages and PMN's as a glycosylphoshatidylinositol-anchored molecule (GPI) membrane CD14 (mCD14) or is present in the circulation as a soluble molecule (sCD14) and lacks the GPI structure (Bazil 1989). It was discovered in 1990 by Wright, et al, and has also been implicated in cell activation processes that involve other products of microbial pathogens. Studies have found that CD14 functions solely as a ligand-binding protein, it does not participate in the generation of transmembrane signals and few details are known about the structure of the ligand binding pocket of this protein (Delude 1995). However, it was hypothesized that one or more additional transmembrane proteins acted together with LPS-CD14 complexes to initiate the signaling processes leading to cell activation. Identification of this transmembrane protein was unsuccessful until the discovery of the Toll-like receptors (TLRs). It has been clearly shown that LPS signal transduction begins on the cell surface by cells that have abundant CD14 and TLR4/MD-2 (Visintin 2003).



Martin et al, Biochim Biophys Acta,2002

Toll-like Receptors

Toll (or TLRs) is the human homologue of the Drosophila Toll which was successfully cloned in 1997 (Medzhitov). They are type-1 transmembrane proteins with their cytoplasmic domain very similar to the human IL-1 receptor. The cytoplasmic domain also contains a signaling domain known as Toll-interleukin (IL)-1 receptor (TIR) domain. The TIR domain is common to all TLRs as well as the IL-1 receptor family (Miyake 2004).



Martin, et al. Biochim Biophys Act 2002

TLR2 was first identified as the main LPS receptor (Yang 1998); however, further studies revealed that these reports were flawed. LPS preparations used in these experiments were contaminated with bacterial lipoproteins which activate TLR2 (Hirschfeld 2000). It is now known that TLR4 is the primary TLR responsible for responses to LPS. C3H/HeJ mice, which are insensitive to LPS, were shown to have a single point mutation in the TIR domain of TLR4 and another LPS-insensitive mouse strain, C57BL/10ScCr was shown to have a null point mutation in the TLR4 gene thus providing the evidence that TLR4 was the receptor for LPS (Poltorak 1998). However, recent studies have indicated that other types of LPS, in particular LPS of Leptospira interrogans (Werts 2001), Porphymonas gingivalis (Bainbridge 2001), and Neisseria meningitides (Pridmore 2001) involve TLR2 for cell activation.

TLR4 works downstream of CD14 , and delivers the LPS signal (Miyake 2004). There are four TIR-domain-containing adapter molecules that mediate the signaling

of TLR4, with all four sharing significant similarity of their amino acid sequencing in their TIR domain: 1. Myeloid differentiation factor 88 (MyD88) (Muzio 1997). 2. MyD88 adapter like (Mal), also called TIR-domain-containing adapter protein (TIRAP) (Fitzgerald 2001). 3. TIR-domain-containing adapter inducing interferon-B (TRIF), also called TIR-domain-containing adapter molecule-1 (TICAM-1) (Yamamoto 2002). 4. TRIF-related adapter molecule (TRAM), also called TIR-containing protein (TIRP) or TIR-containing adapter molecule (TICAM-2) (Oshiumi 1998). Unlike other TLR's that use some of these adapter molecules, TLR4 requires all four to initiate a comprehensive immune response. (Fitzgerald 2004). It is currently thought that the propagation of LPS signaling is achieved following the recruitment of these four TLR-domain containing adapter molecules. They provide a structural platform that activate downstream kinases, which ultimately leads to the activation of specific transcription factors and inflammatory gene expression.

MyD88 was first identified as a gene that was induced during IL-6 stimulated differentiation of M1 myeloleukemic cells into macrophages and then cloned as a TIR and death domain (DD)–containing protein . It is recruited to the C-terminal TIR domain of the IL-1R and then recruits the serine/threonine kinase IL-1R-associated kinase-1 (IRAK1) through its N-terminal DD (Muzio 1997). Not only does it interact with the IL-1R, it is brought to the TIR domain of other TLR families with the exception of TLR3; therefore, MyD88 is a shared component of IL-1R/TLR signaling (Kawai 1999). Mice, deficient in MyD88, were insensitive to LPS-induced death and failed to release IL-6 and tumor necrosis factor alpha (TNF-α) in vivo, or in LPS-stimulated macrophage cultures in vitro (Kawai 1999). The existence of an MyD88

independent pathway to signal TLR4 was also identified after nuclear factor kappa beta (NFkβ) translocation and phosphorylation of mitogen-activated protein kinases occurred in these MyD88 deficient mice, although delayed, it was not eliminated (Kawai 1999). Unpublished results suggest that MyD88-independent signaling may be responsible for the majority of the LPS response (Fitzgerald 2004).

Mal or TIRAP is another TIR-domain-containing protein which mediates the signaling of TLR4 but not IL-1R ((Fitzgerald 2001). It has the C-terminal TIR domain but not the N-terminal DD present in MyD88. Mal-deficient macrophages have severely impaired LPS-induced cytokine production with normal responses of the TLR5,TLR7, TLR9, IL-1 and IL-18 receptors (Yamamoto 2002).

There is no LPS- induced cytokine gene expression in TRIF or TICAM-1 deficient macrophage cultures; however, there is normal cytokine expression via TLR2, TLR7 and TLR9 (Fitzgerald 2003).

TRAM associates with TLR4 and TRIF. It has been shown that TRAM and TRIF regulated MyD88 independent signaling (Fitzgerald 2003). Studies of TRAM-deficient mice show that TRAM is essential for the MyD88 independent signaling pathway (Yamomoto 2003). TRAM-deficient mice also have defective induction of inflammatory cytokines from LPS (Fitzgerald 2003).

Although TLR4 is essential for LPS signaling, studies suggested that an additional molecule was required. Human embryonic kidney (HEK)-derived 293 cells and mouse IL-3 dependent pro-B cell line Ba/F3 did not have the ability to respond to LPS after in vitro transfection of TLR4 cDNA (Shimazu 1999). This

unresponsiveness was explained through the discovery of an additional component of the LPS recognition complex, MD-2 (Shimazu 1999).

MD-2

MD-2 is an 18 kDA protein that is bound to TLR4 in the Golgi and also found as a soluble molecule that is secreted by MD-2 expressing cells (Visintin 2001). It is absolutely necessary for LPS recognition and it must be physically associated with TLR4 for this recognition to occur (Miyake 2004). The association between these two proteins occurs in the endoplasmic reticulum (ER) and studies have also shown that gp96, an ER chaperone, plays an important role (Visintin 2001). The exact binding site of TLR4 to MD-2 is not known, and neither is the exact location of the LPS to MD-2 binding site (Miyake 2004). Some of the amino acids crucial for MD-2 interaction with TLR4 have been identified (Visintin 2003). It has been shown in vitro that cells which express TLR4 alone or with a mutant MD-2, are hyporesponsive to LPS (Shimazu 1999). It has also been shown that a lack of response to LPS can be altered with transfection of MD-2 cDNA or soluble MD-2 protein (Schromm 2001).

In vitro studies using cells that lack mouse MD-2 have shown an important role for MD-2 in TLR4 cell surface expression. Murine fibroblasts that were MD-2 deficient, temporarily expressed mouse TLR4 stacked in the Golgi apparatus. In wild-type fibroblast cells, TLR4 moved beyond the Golgi apparatus to the cell surface. These same results have also been described for human MD-2 (Nagai 2002). Gp96 has also been shown to play an important role in the expression of TLR4-MD-2 on the cell surface Gp96-deficient cells do not form the TLR4-MD-2 heterodimer (Randow 2001).

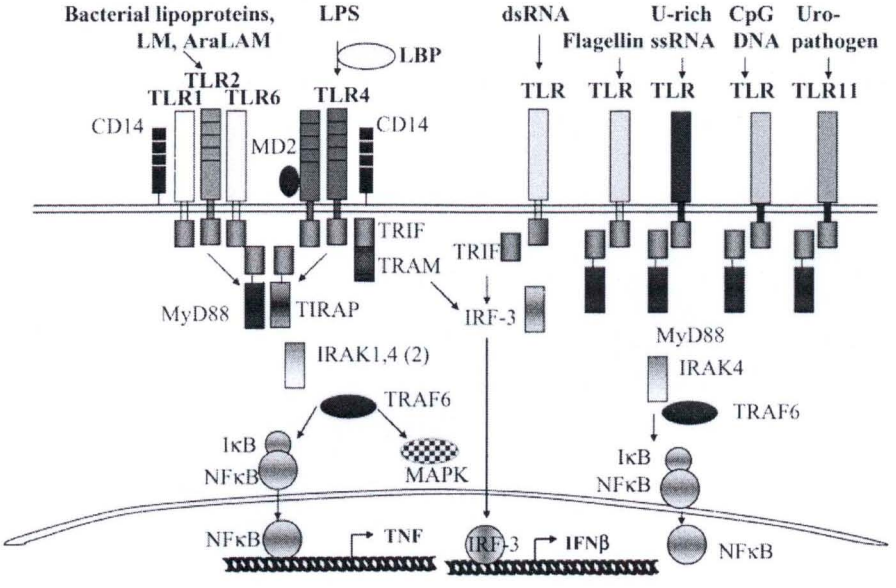
LPS Signal Transduction

It is thought that LPS activation of nuclear factor kappa beta (NFκβ), a group of transcription factors necessary for innate and adaptive immunity, via TLR4 occurs through a signaling pathway that is also utilized by IL-1 (Yang 2000). The binding of MyD88 to the TIR domain of TLRs mediates their signal transduction. MyD88 contains a C-terminal Toll homology which interacts with the Toll domain of the receptor, and an N-terminal death domain. Its death domain interacts with the death domain of an IL-1 receptor associated kinase (IRAK), leading to the autophosphorylation of IRAK (Aderem 2000). IRAK can now complex with an adaptor, TNF receptor associated factor (TRAF) 6 which then activates transforming growth factor beta (TGFβ)-activated protein kinase-1 (TAK-1), a member of the mitogen-activated protein (MAP) kinase family, though the details of this pathway are less clear (Aderem 2000). The activation of TAK-1 activates the I κβ kinases (IKK) which in turn phosphorylates the inhibitory proteins, IKβ, causing their proteolytic degradation and the translocation of NF-κβ to the nucleus (Aderem 2000) where transcription is activated. The role of MyD88 and TRAF6 in LPS signaling have been confirmed genetically with the inability to respond to LPS in MyD88-deficient mice (Kawai 1999). TRAF-6 deficient mice are also hyporesponsive to LPS (Lomaga 1999). This data, taken together, indicates a role in LPS signaling in monocytes and macrophages for MyD88, IRAK and TRAF6 (Li 2000).

IKKα and IKKβ are the two kinases (IKK) responsible for phosphorylating Ikβ's (May 1999) and they form a large multiprotein complex with NEMO (IKK γ), a scaffold protein (Yamaoka 1998). The activation of these two kinases is thought to

be through phosphorylation by an upstream kinase. NF-κβ inducing kinase (NIK) and mitogen-activated protein kinase ERK kinase kinase 1 (MEKK)) are both candidates for this role because each of these can activate NF-κβ through phosphorylation and activation of the IKKs (Lee 1997). In addition, LPS activation of NF-κβ and TNF-α promoter activity in mouse RAW 264.7 cells was inhibited in dominant negative mutants of NIK, MEKK1, and TAK-1 (Swantek 1999). It has been shown that LPS activates the IKKs in human monocytes, THP-1 monocytic cells and mouse RAW 264.7 cells (O'Connell 1998). Activation of IKK's by LPS was relatively slow - peaking at about thirty minutes – when compared with TNF-α activation of the IKKs – peaking at about five minutes. This suggests involvement of different signaling pathways by these two agonists (Hawiger 1999). Further study has shown that LPS induction of NF-κβ gene expression and TNF-α promoter activity is inhibited by a dominant-negative mutant of IKKβ and that a dominant-negative mutant of IKKα had no effect (O'Connell 1998). Knock-outs of IKKα genes result in abnormal morphogenesis, whereas knock-outs of IKKβ genes results in embryonic lethality caused by severe liver degeneration during midgestation, a phenotype consistent with excess TNF-α toxicity (Beg 1995). From these results and from studies using IKK-αand IKK-β deficient fibroblasts, it was concluded that IKKβ but not IKKα was required for TNF-αand IL-1 signaling and that IKK-β is required for

LPS activation. (May 1999).



Quesinaux et al. Microbes and Infections 6 (2004)

The IL-1 receptor associated kinases, IRAK (Cao et al 1996), IRAK-2 (Muzio et al 1997), IRAK-3 (Wesche et al., 1999) , and IRAK-4 (Li 2002) comprise the serine/threonine kinase family. They were first described in 1994 (Martin 1994), cloned in 1996 (Cao 1996), and are important mediatiors of Toll/IL-1 receptor family signal transduction (Kollewe 2002). IRAK and IRAK-4 are active kinases, and IRAK-2 and IRAK-3 are the two inactive or inhibitory kinases . IRAK-3 is found only on macrophages/monocytes. When stimulated by TLRs, IRAK is recruited to the TLR signaling complex (Medzhitov 1998).

Osteoblasts

Osteoblasts are bone forming cells that originate from mesenchymal tissue and are nearly indistinguishable from fibroblasts in cell culture. Mature osteoblasts, which are aligned on the bone surface, are responsible for the secretion and deposition of most

of the bone matrix proteins such as type I collagen, osteocalcin, osteonectin, osteopontin, bone sialoprotein and proteoglycans. Another component of bone, hydroxyapatite crystals in osteoid, is also regulated by osteoblasts. Therefore, major functions of osteoblasts include: the expression of a number of bone-related extracellular matrix proteins, high enzyme activity of alkaline phosphatase (ALP), and responses to osteotropic hormones and cytokines (Katagiri 2002).

Osteoblast differentiation can occur through two distinct pathways during embryonic development: endochondral ossification or intramembranous ossification (Horton 1993).

Ostoblasts differentiate directly from mesenchymal cells during intramembranous ossification. During endochondral ossification, chondrocytes differentiate from mesenchymal cells and form a cartilaginous template. After maturation of hypertrophic chondrocytes in the template, osteoblasts are immediately formed from the surrounding mesenchymal cells (Chung 1998). The processes of bone and cartilage formation may mean that theses tissues are derived from a common progenitor cell. Calvaria or bone marrow cell cultures showed mixed populations of osteoblasts, chondrocytes, adipocytes and skeletal muscle cells (Taylor 1979). The formation of these tissue-specific cells indicate that a pluripotent progenitor is capable of differentiation (Grigoriadis 1998). The mineralized extracellular matrix is the only morphological feature specific to osteoblasts; however, the genes responsible for this matrix have not been found to be selectively expressed in osteoblasts (Ducy 2000).

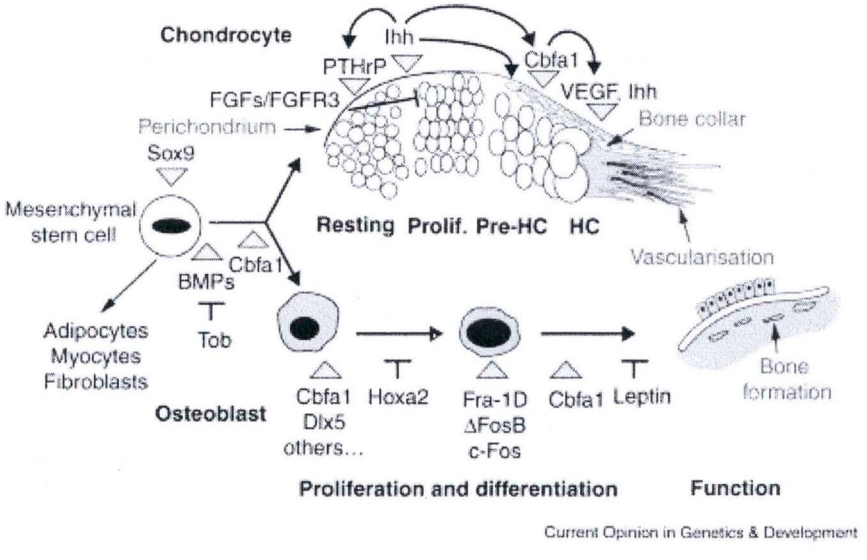
Regulation of osteoblast differentiation

In the search for the "master" regulators of ostoblast differentiation, two osteoblast-specific cis-acting elements (OSEs) were identified in the promoter of the Osteocalcin gene (*Bgp*): OSE1 and OSE2. Cbfa1, which binds to OSE2, was then identified as the first osteoblast-specific transcription factor (Ducy 1997). It is an important and necessary gene for osteoblast differentiation and is one of the earliest and most specific markers of osteogenesis (Ducy 2000). It is homologous to one of the three Drosophila runt and lozenge proteins ((Speck 1995). Cbfa1 expression precedes osteoblast differentiation during embryonic development and it is restricted to mesenchymal cells that will eventually become chondrocytes or osteoblasts (Ducy 1997). Its expression subsequently becomes limited to osteoblasts, with a lower level of expression in hypertrophic chondrocytes (Kim 1999). Odontoblasts, dentin-synthesizing tooth homologs of osteoblasts, also express Cbfa1 (D'Souza 1999).

Cbfa1 is an activator of transcription and induces osteoblast-specific gene expression in fibroblasts and myoblasts (Ducy 1997). Mice, deficient in Cbfa-1, develop full term with a normal skeleton made exclusively of cartilage: osteoblast differentiation never occurs (Komori 1997), and certain bones show defects in hypertrophic chondrocyte differentiation (Kim 1999). These mice also lack osteoclasts because osteoblasts are required for osteoclast differentiation. Upstream expression control of Cbfa1 has not been identified. Gene deletion experiments in mouse models, suggest that it is controlled in the developing skeleton by different transcription factors at different locations. It is also not known what transcription factors act downstream of Cbfa1 (Ducy 2000).

OSE1, the other cell–specific regulatory element in the *Osteocalcin* promoter, is as important as the Cbfa1 binding site for *Osteocalcin* expression (Schinke 1999). The factor that binds OSE1, has been biochemically characterized recently, but its complete identification will help to further define the genetic pathways controlling osteoblast differention (Ducy 2000). It is also possible that new transcription factors important for osteoblast differentiation may be identified through broad-based gene deletion experiments (Ducy 2000).

Osteoblast differentiation during embryonic development involves members of all the major families of growth factors. Expression of Cbfa1 can be induced in vitro by several bone morphogenetic proteins; however, it appears to be through indirect action and is of unknown physiological relevance (Ducy 1997). In cultured osteoblasts, the steady state level of osteoblast differentiation in vivo and inhibition of Cbfa1 expression can be controlled by transforming growth factor- beta (TGF-β). The mechanism through which this occurs is unknown (Ducy 2000).



Wagner, et al (2001)

Bone Morphogenetic Proteins

When pulverized bone organic matrix is implanted into muscular tissue it induces ectopic bone formation as was described by Urist in 1965 (Urist 1965). These proteins were eventually named 'BMPs" after their cDNA was isolated (Wozney 1988). Ectopic bone formation has been shown to be induced by BMP proteins (Kingsley, 1994) and this activity is unique to BMPs among the growth factors. BMPs, except BMP-1, belong to the TGF-β family of growth factors, regulate the proliferation, differentiation and death of cells in various tissues (Hogan 1996), and are believed to be secreted and deposited by osteoblasts into the extracellular matrix during bone formation.

The hypothesis that BMPs regulate osteoblast and chondrocyte differentiation during skeletal development was confirmed with the identification of skeletal abnormalities in animals and patients with mutations in the BMP genes. Mutation in BMP-5, the mutant mouse 'short ear' was the first example to be shown by Kingsley in 1992 (Kinglsey 1992). He showed that the BMP-5, which is deleted or rearranged in several independent mutations at the short ear locus, was encoded in the short ear region. However, whether BMPs are involved in bone and cartilage formation after birth is still unclear (Katagiri 2005).

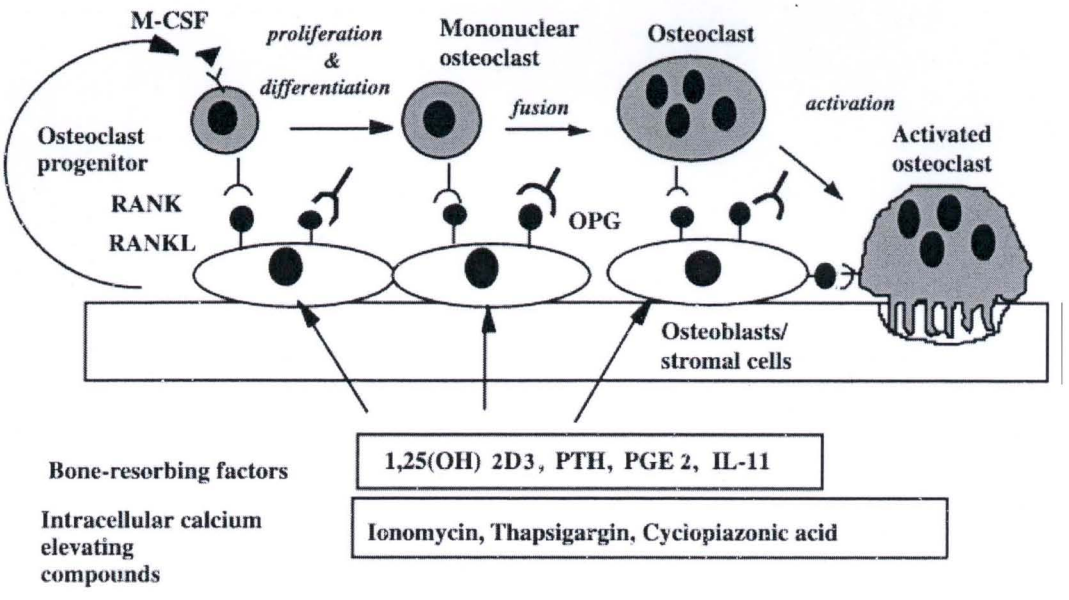
Transcriptional control of bone formation

In addition to its role during osteoblast formation, Cbfa1 also controls bone formation. It regulates the expression of Osteoclacin (*Bgp*), a gene that is expressed only in terminally differentiated osteoblasts (Ducy 2000). Genes required for the formation of bone extracellular matrix contain cbfa1 binding sites (Ducy 1997) and

transgenic mice experiments show that the rate of bone formation by differentiated osteoblasts are controlled by Cbfa1 (Ducy 1999).

Osteoblasts regulate osteoclast differentiation

Osteoclast development occurs within the microenvironment of bone in response to a variety of osteotropic factors including 1,25-dihydroxyvitamin D_3 $(1,25OH)_2D_3$, PTH, prostaglandin E_2 (PGE_2) and interleukin 11 (IL-11). The earliest identified osteoclast progenitor in bone marrow has been phenotyped as a c-Kit+/c-Fms-/CD11b-/RANK- cell which then differentiates into a c-Kit+/c-Fms+/CD11b+/RANK- early stage progenitor (Boyce 2005) This cell, termed a late stage progenitor, then becomes RANK+ upon stimulation by M-CSF. It responds to RANKL to complete osteoclast development (Boyce 2005). To investigate the regulatory mechanisms of osteoclast differentiation, osteoblasts/stromal cells and hematopoietic cell cocultures were developed (Takahashi 1988). The multinucleated cells that formed expressed tartrate-resistant acid phosphatase (TRAP – a marker enzyme of osteoclasts), calcitonin receptors, vitronectin recepors and the ability to form resorption pits on bone and dentine slices (Katagiri 2002). Osteoclastogenesis requires cell-to-cell contact between osteoblasts/stromal cells and osteoclast progenitors (Jimi 1996). Osteoblast/stromal cells are the targets of osteotropic factors to induce osteoclastogenesis (Udagawa 1995).

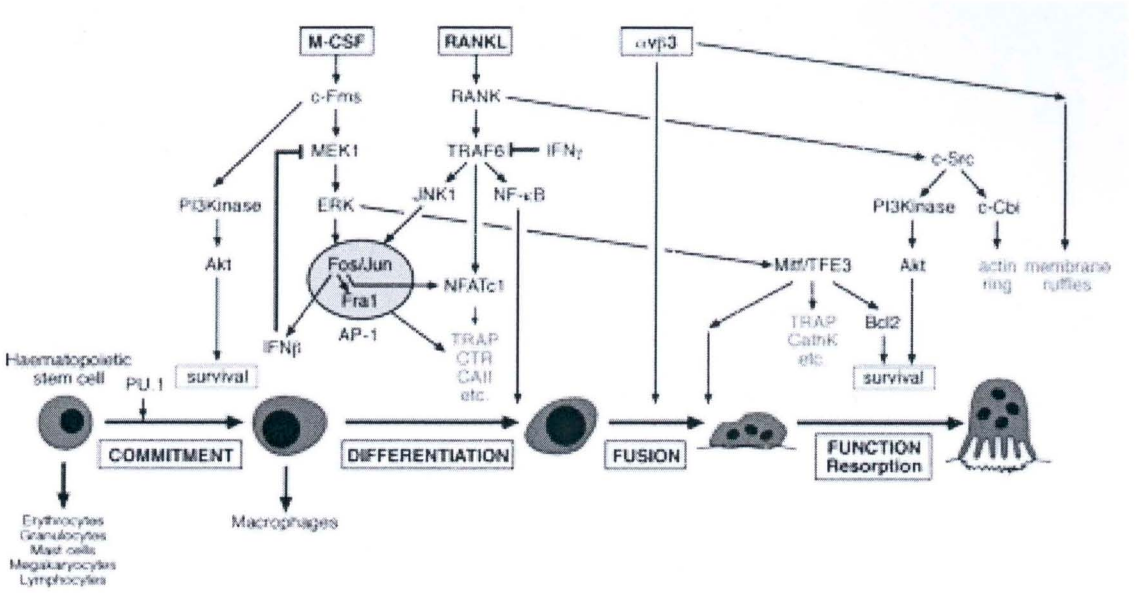


Katagiri and Takahashi (2002) Oral Diseases

Osteoclasts – Origin and Cell Lineage

Osteoclasts, tissue-specific macrophage polykaryons, are created from monocyte/macrophage precursor cell differentiation at or near the bone surface (Boyle 2003). Their hematopoietic origin was demonstrated in transplantation studies (Croccia 1980). In vivo (Baron 1986) and in vitro studies (Schneider1988) initially suggested that osteoclasts were derived from mononuclear phagocytic cells; however, although macrophages and osteoclasts share a common precursor, these lineages diverge upon further differentiation (Hagenaars 1989). Compared to cells of the macrophage series, they possess a distinct phenotype and functional capabilities, in particular, resorbing bone (Zhao 2007). Our understanding of osteoclastogenesis was advanced when co-cultures of bone marrow or spleen cells and stromal cells from murine systems yielded osteoclasts (Takahashi 1988). It was determined that

stromal-derived factors stimulated this process because of the need for close contact between osteoblasts/stromal and bone marrow cells, and it is now known that these cells express the polypeptide growth factor, macrophage colony-stimulating factor (M-CSF) and the cytokine IL-6 that induce and control growth and differentiation of the precursors to mature osteoclasts (Burger 1984). Calcitrol, the vitamin D metabolite, and the parathyroid hormone also support development of osteoclasts (Suda 1992). Multiple Golgi complexes and numerous mitochondria are present in the osteoclast precursor cells (Baron 1986). "Preosteoclasts", a term which is used to describe a mononuclear precursor that shows morphological and cytochemical features similar to those of multinucleated osteoclasts, will differentate from osteoclast precursors in a suitable microenvironment (Scott 1967). Ready to fuse into multinucleated osteoclasts upon further differentiation, these cells are TRAP-positive and express mRNAs for all osteoclast-associated phenotypes which include: TRAP, calcitonin receptor (CTR) and cathepsin K (Scheven 1986). A highly specialized proton-generating pump found in osteoclasts rapidly dissolves minerals, secretes collagenases, (Wucherpfennig 1994) cathepsin K (Li 1995)and other hydrolases involved in the degradation of bone matrix proteins. M-CSF and RANKL are both needed to induce gene expression of the osteoblast lineage which leads to mature osteoclast development (Lacey 1998).



Wagner and Matsuo, Ann Rheum Dis (2003)

Osteoclast gene transcription

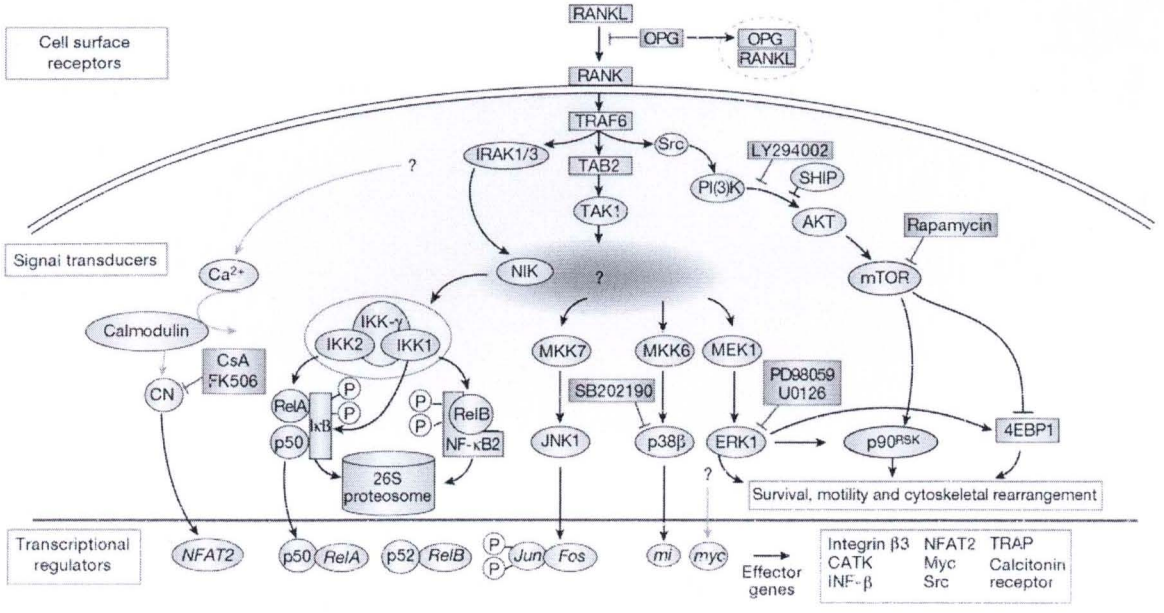
It has been shown that at least twenty-four genes or loci positively and negatively affect osteoclastogenesis and osteoclast activation (Boyle 2003). These findings are based on naturally occurring mutations or targeted knockout mutations in humans and rodents which show blocked development and/or function of the mature osteoclast resulting in osteopetrosis when these genes are disrupted (Mark 1998). The effects of these genes are expressed at different stages of osteoclast development and activation, and the gene products for some these loci have still not been identified (Boyle 2003) The genes PU.1 and op/CSF-1 act during the formation and survival of the osteoclast precursor cell. Essential for the development of myeloid and lymphoid lineage cells, PU.1 is a EST-domain transcription factor. PU.1 is thought to regulate lineage-specific cytokine receptor genes, such as M-CSF receptor (c-fms), GM-CSFRalpha, G-CSFR, and IL-7Ralpha (Singh 1999).

The earliest established event in osteoclastogenesis is regulated by PU.1. The expression of PU.1 is detected at all stages of osteoclast differentiation. As cells differentiate into osteoclasts, PU.1 mRNA increases 3-fold (Tondravi 1997) Embryonic stem (s) cell disruption of PU.1 causes lack of macrophages and osteoclasts with osteopetrosis (Tondravi 1997). Corresponding sequences in the promoters and enhancers of many osteoclast specific genes are bound to by PU.1. The RANK gene is a transcriptional target of PU.1. Failure of expression of the RANK gene is seen in PU.1-/- progenitor cells; however, reconstitution of PU.1 induced RANK expression (Kwon 2005). PU.1 and NFATc1 together regulate RANKL-induced cathepsin K gene expression (Matsumoto 2004). Tartrate-resistant acid phosphatase gene expression is activated in osteoclasts due to the interaction of micropthalmia associated factor (Mitif), a transcription factor, and PU.1 with the tartrate-resistant acid phosphatase (TRAP) gene promoter (Partington 2004). Therefore, PU.1 is a master regulator that is critical for development of a common progenitor for the lymphoid myeloid cell lineages in the hematopoietic system (Zhao 2007).

Receptor activator of NFκB (RANK) Signaling and Osteoclastogenesis

RANK, the receptor for RANKL, is a member of the tumor necrosis factor (TNF) receptor family and is involved in osteoclastogenesis and lymph node development (Anderson 1990). It is expressed as a transmembrane heterotrimer on the surface of hematopoietic osteoclast progenitors, mature osteoclasts; chondrocytes and mammary gland epithelial cells (Anderon 1997). RANKL interacts with RANK via direct cell to cell contact, thereby promoting the differentiation, survival, and bone-resorbing

capability of osteoclasts (Suda 1999). It has been shown that mice, genetically deficient in either RANK or RANKL show a profound defect in osteoclastogenesis which results in severe osteopetrotic phenotype (Kong 1997). Cytokines such as M-CSF and RANKL that initiate and control the growth and differentiation of the precursors to mature osteoclasts are produced by osteoblasts/stromal cells (Zhao 2007). The binding of M-CSF to its receptor, c-Fms, present on osteoclast precursors provides the macrophage survival and proliferation signals. The expression of osteoclast-specific genes during differentiation, the activation of resorption by mature osteoclasts, and their survival and participation in bone degradation at neighboring sites is activated by the RANK/RANKL interaction (Boyle 2003). Cytoplasmic factors that activate downstream signaling pathways that control these various functions mediate RANK signaling. At least five distinct signaling cascades mediated by protein kinases are induced during osteoclastogenesis and activation – inhibitor of NF-κβ kinase (IKK), c-Jun N-terminal kinase (JNK), p38, extracellular signal-regulated kinase (ERK) and Src pathways (Boyle 2003). (BOYLE DIAGRAM)



Boyle (2003) Nature

Since RANK lacks intrinsic enzymatic activity in its intracellular domain, it transduces signaling through the recruitment and activation of cytoplasmic tumor necrosis factor receptor-associated factors (TRAFs) (Asagiri 2006). The binding of TRAFs to specific cytoplasmic domains within RANK is the key preliminary step in RANK signaling (Hsu 1999). TRAF2, -5 and -6 have all been shown to bind to RANK; however, only TRAF6 mutations result in osteopetrosis due to a loss of osteoclast activity (Lomaga 1999).

The RANK signaling pathway has several levels of control that enhance or dampen osteoclastogenesis and activation driven by RANKL (Boyle 2003). *In vitro* activation of osteoclast surface receptors for IL-1, c-Fms, TNF-α, PGE2 and TGF-β potentiate osteoclastogenesis, and can stimulate bone resorption *in vivo* (Boyle 2003). Signaling through TRAF-6 by IL1-R and TNFR1, and activation of these receptors could have a synergistic effect on RANK-mediated TRAF6 activation (Mak 2002). It has been reported that the activation of c-Fms and TGF-β up-regulates components of

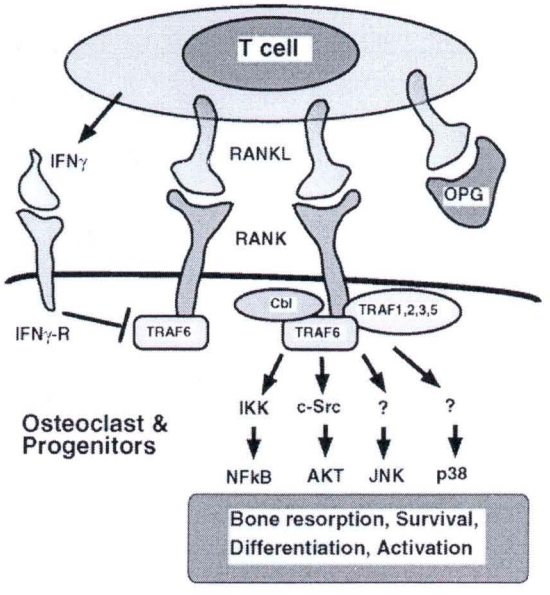
the pathway, including the levels of RANK on the cell surface, thereby impacting the potency of the RANKL in this system (Arai 1999). Osteoprotegerin (OPG) negatively controls the RANK signaling pathway *in vitro and in vivo* (Simonett 1977). Evidence exists for feedback mechanisms that switch off the RANK signaling pathway once it is activated. Secretion of interferon-β due to activation of osteoclastogenesis by RANKL acts in an autocrine manner to downregulate the expression of c-Fos, a critical factor involved in osteoclast development (Takayanagi 2002).

Receptor activator of NFκβ ligand (RANKL)

RANK and RANKL are two members of the TNF family that are primary regulators of bone remodeling and are necessary for the development and activation of osteoclasts. The *rankl* gene was cloned by four independent groups at the same time (Anderson 1997) (Lacey 1998) (Wong 1997) (Yasuda 1998). This gene encodes a molecule of 316 amino acids, and three RANK-L subunits which assemble to form a trimeric molecule (Theill 2002). Disruption of this gene in mice causes severe osteopetrosis, tooth eruption impairment, and osteoclast absence (Kong 1999). Osteoblast/stromal cells and primitive mesenchymal cells that surround the cartilagionous anlagen, hypertrophied-chondrocytes and activated T cells express RANKL which is a membrane bound factor. RANKL is made by T cells following antigen-receptor stimulation (Theill 2002). RANKL's discovery helped establish that osteoblasts/stromal cells support osteoclast differentiation primarily by serving as a source of RANKL as well as M-CSF (Suda 1999). M-CSF and RANK bind to their respective receptors, c-fms and RANK expressed on osteoclast precursors to

stimulate formation of osteoclasts. M-CSF and RANKL have been shown in vitro to be sufficient for osteoclastogenesis (Quinn 1998). RANKL also mediates activation and survival of mature osteoclasts (Lacey 1998). Through investigation of the RANK/RANKL system, many osteotropic hormones and cytokines have been revealed that regulate osteoclast formation and function through modulating RANKL expression by osteoblasts/stromal cells (Hofbauer 2000).

Molecules that regulate RANKL include PTH (Lee and Lorenzo, 1999), vitamin-D_3 (Miura et al., 2002), TNF-α (Hofbauer et al., 1999b), glucocorticoids (Chung et al 2001), PGE_2 (Li 2002), interleukin-1 and -11 (Hofbauer 1999b), thyroid hormone (Miura 2002), lipopolysaccharide (Kikuchi 2001), fibroblast growth factor-2 (FGF-2) (Chikazu 2001), insulin like growth factor-1 (IGF-1) (Rubin 2002), TGF-β and BMP-2 (Theill 2002) and low gravity (Kanematsu 2002). T cell/dendritic communications, dendritic cell survival, and lymph node organogenesis are also regulated by RANK-L (Theill 2002). Activated T cell production of RANK-L can regulate osteoclastogenesis and bone remodeling. RANK and RANK-L also have an essential role in the formation of a lactating mammary gland during pregnancy (Theill 2002).



Theill, et al. Annu Rev Immunol 2002

Osteoprotegerin (OPG)

Osteoprotegerin (OPG), homolgous to TNF receptor family members, is a secreted protein which serves as a soluble decoy receptor to RANK-L and competes with RANK for RANK-L binding making it an in vitro inhibitor of osteoclast maturation and activation (Simonet 1997). OPG is expressed in the stromal cell line and human bone marrow stromal cells, is down regulated by bone resorbing factors vitamin D_3, prostaglandin E_2 (PGE_2) and glucocorticoids, and upregulated by calcium ions and TGF-β (Brandstrom 1998). Osteopetrosis results from the overexpression of OPG in transgenic mice (Simonet 1997).

Developmental regulation, activation of osteoclasts and bone metabolism experiments by different groups show that there is a balance between RANK-L-RANK signaling and biologically active levels of OPG (Theill 2002). This complex system of bone remodeling regulated by osteoclasts appears to be controlled by these

three molecules (Theill 2002). However, osteoclast formation and activation is also controlled by genes, hormones and cytokines.

TABLE 1 Molecules that regulate RANK-L and OPG levels

	RANKL	OPG
Hormones		
Vitamin-D3	Increased	Increased
PTH	Increased	Decreased
PTHrP	Increased	Decreased
Estradiol	No change	Increased
Cytokines		
TNF-α	Increased	Increased
IL-1	Increased	Increased
IL-6	Increased	n.t.
IL-11	Increased	n.t.
IL-17	Increased	n.t.
Growth factors		
TGF-β	Decreased	Increased
BMP-2	n.t.	Increased
Others		
Prostaglandin E2	Increased	Decreased
Glucocorticoid	Increased	Decreased
CD40L	Increased	n.t.

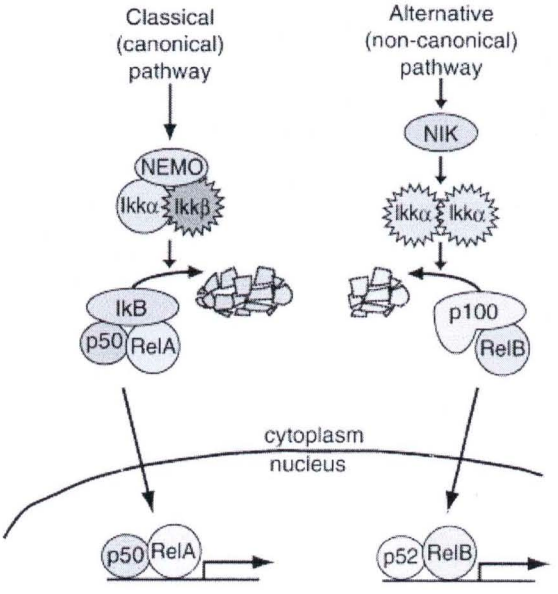
Theill, et al. Annu Rev. Immunol 2002

Tumor Necrosis Factor Receptor-Associated Factors (TRAF's)

The TRAF family consists of seven distinct proteins and serve as cytoplasmic adapters that can interact directly with the intracellular domains of cell surface receptors, such as the TNF receptor family, and mediate signaling (Asagiri 2006). TRAF-6 is the essential adaptor required for RANK-associated signaling and is the only member of the Toll/IL-1R family to have interaction with 1L-1R associated kinase (IRAK). It binds to the membrane-distal domain of RANK's cytoplasmic tail (Cao 1996). As mentioned earlier, TRAF-2 and -5 have also been shown to bind to

RANK; however, osteopetrosis due to a loss of osteoclast activity, only results when TRAF-6 is blocked (Lomaga 1999). TRAF-6 acts as a key adaptor to assemble signaling proteins that direct osteoclast-specific gene expression leading to differentiation and activation. The activation of transcription factors NF-κβ and activator protein-1 (AP-1) are the two most closely studied pathways involved with TRAF-6 since their activities are rapidly induced following ligand-binding (Boyle 2003).

One of the most important and earliest pathways activated by RANK is NF-κβ. This pathway is relevant for RANKL-RANK regulated osteoclast development and osteoclast function in mice and humans (Doffinger 2001). Although TRAF6 is critical for the RANK-induced activation of NF-κB, it is unlikely that NF-κB is the only downstream molecule mediating the complex functions of TRAF6 (Asagiri 2006). However, among the molecules immediately activated by TRAF6, genetic evidence supports the essential role of NF-κB, but not that of other molecules, such as mitogen-activated protein kinases (MAPKs), in osteoclastogenesis (Asagiri 2006). NF-κB activation depends on two pathways: the classical and alternative (Asagiri 2006). The classical pathway involves the IKK complex as described earlier which involves activation of the inhibitor of the κB (IκB) kinase (IKK) complex that phosphorylates the IκBs and targets them for ubiquitin-dependent degradation (Asagiri 2006). The alternative pathway is responsible for activation of the p52:RelB dimers, which are generated by the processing of the cytoplasmic p100:RelB complex (Asagiri 2006).



Asagiri et al, Bone 2007

Nuclear factor kappa beta

Nuclear factor kappa beta (NFκβ) is a collective name for a group of transcription factors that are necessary for innate and adaptive immunity. They are members of the Rel family of transcriptional activator proteins and are found in the cytoplasm of most cells (Thanos 1995). They were first described in 1986 as a nuclear factor in B cells that bound a site in the immunoglobulin κ enhancer (Sen 1986). It was soon shown that their activation in other cells occurred by exposure to stimuli such as phorbol esters (Sen 1986) and in the following years, functional binding sites for NF-κβ were found in the promoters of many genes (Baldwin 1996). NF-κβ binding sites usually act as transcriptional regulatory factors that induce a wide variety of genes involved in immune function, inflammatory response, cell adhesion and growth control (Baldwin 1996) such as pro-inflammatory cytokines TNF-α, interleukin 1 and-6, chemokines, RANTES (regulated upon activation, normal T-cell expressed and secreted) and vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (Zhang

2001). Microbiocidal effector molecules and enzymes that create intermediates such are nitric oxide are also induced by NF-κβ (Zhang 2001). T and B cell activation (Li 2002) and regulation of peripheral lymphoid organogenesis is also induced by NF-κβ (Weih 2003). Its activation is not only limited to mediators of immune function, other stimuli such as UV irradiation, growth factors and viral infection activate NF-αβ (Baldwin 1996).

There are five NF-κβ proteins expressed in mammals which are made up of complexes of Rel family polypeptides and are divided into two different groups based on structure, function and mode of synthesis (Siebenlist 1994)). Subunits p50 (NF-κβ1) and p52 (NF-κβ2) comprise the first group and are synthesized as precursor proteins (Thanos 1995). They become active when they are processed proteolytically (Xiao 2001). The second group which are not synthesized as precursors, include p65 (Rel A), RelB, and c-Rel (Beinke 2003). Rel-A and c-Rel can activate transcription from target gene NF-κβ binding sites and Rel-B can complex with p50 or p52 to function as an NF-κβ activator (Beinke 2003). RelB/RelA complexes cannot bind DNA so they are inhibitory. p50 and p52 subunits can only promote transcription when heterodimerized with a transactivated Rel subunit. (Beinke 2003)

Due to a defect in osteoclast differentiation, mice lacking NF-κβ1 and NFκβ2 develop osteopetrosis which suggests a redundant role of NF-κβ1 and NF-κβ2 (Iotsova 1997). This suggests that early stage arrestment of osteoclast differentiation is attributed to the complete lack of osteoclasts in these mice. NF-κβ1 and NFκβ2 are essential for RANK-expressing osteoclast precursors to differentiate, in response

to RANK and other osteoclastogenic cytokines, into TRAP+ osteoclasts; however, they are not required for formation of RANK-expressing osteoclast progenitors (Xing 2002).

NF-κβ Activation Pathway

NF-κβ dimers are inactive and sequestered in the cytoplasm of unstimulated cells. Inhibitors of NF-κβ (Ikβ's) are the proteins responsible for keeping NF-κβ dimers in a cytoplasmic quiescent state (Verma 1995). Stimuli such as LPS, TNF-α and IL-1 cause a signaling cascade which likely leads to activation of multiple upstream kinases and an increase in the activity of the Iκβ kinases (IKK) (Ghosh 2002). Specific serines within the Ikβ's are phosphorylated by the IKK complex which results in their degradation mediated by proteasomes. With the degradation of the Ikβ's, the NF-κβ dimers move to the nucleus to activate target genes (Stancovski 1997).

Studies indicate that phosphatidylinositol 3-kinase (P13K) is a downstream effector involved in liberating NF-κβ from Iκβ (Reddy 1997). It consists of a catalytic (p110) and regulatory (p85) subunits which activate lipid substrates at the cell membrane that act as secondary messengers (Sizemore 1999). LPS stimulation of monocytes/macrophages activates the P13κ pathway (Lee 2000). It is unclear how P13K and its downstream effectors feed into a signal transduction cascade that leads to activation of NF-κβ ((Bergmann 1998).

Current studies demonstrate that LPS stimulation of monocytes leads to an activation of the P13κ-Akt pathway, which inactivates MAPK kinase pathways (ERK ½, p38 and JNK) and the the NF-κβ pathway (Guha). Blockage of these pathways with the use of inhibitors, such as LY294002, limits the activation of the transcription factors NF-κβ, and AP-1 which regulated TNF-α gene expression. The PI3κ-Akt pathway is a control mechanism to limit the expression of TNF-α in LPS stimulated monocytes which only enables transient expression of these inflammatory mediators (Guha 2002)

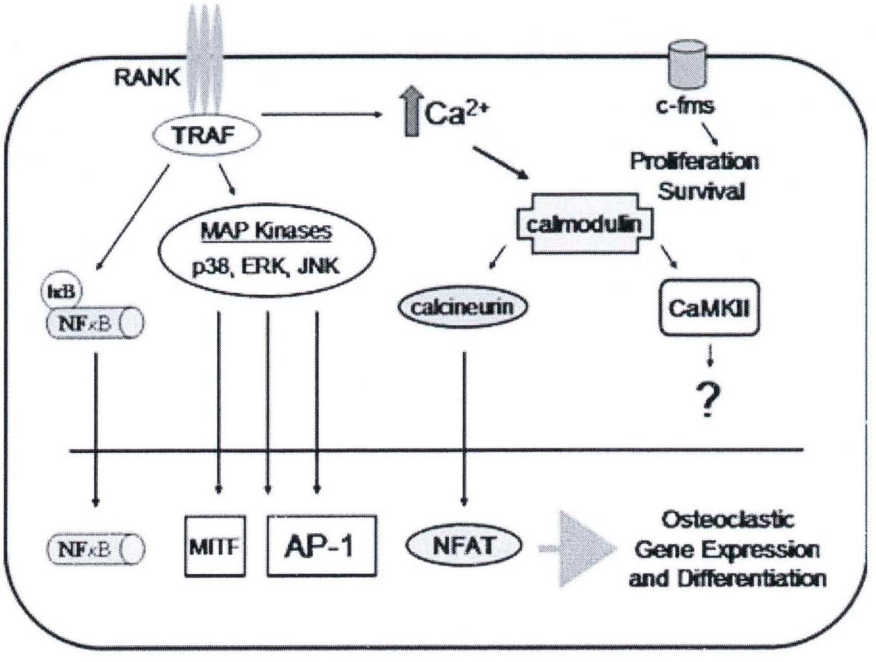
Mitogen-activated protein kinases (MAPKs)

Cell-surface receptors are connected to critical regulatory targets within cells via mitogen-activated protein kinases, which are evolutionary conserved enzymes (Chang 2001). Thrr major subfamilies of MAP kinases have been identified: extracellular signal regulated kinases (ERKs), JNKs and p38 MAP kinases. Peptide growth factors and phorbol esters preferentially activate ERKs, while cellular stresses, such as hyperosmolarity or reactive oxygen species, potently activate JNKs and p38MAP kinases (Iwasaki 1999). A three-tiered cascade regulates their activity and is composed of a MAPK, MAPK kinase (MAPKK, MKK, or MEK) and a MAPKK kinase (MAPKKK, or MEKK) (English 1999). Even though they are distinct in their activation, there is considerable cooperation between these kinases and many substrates are shared between pathways (Cobb 2000). A wide spectrum of cell functions are influenced by this family of kinases including proliferation (Pages 1993), apoptosis (Cross 2000), cytokine biosynthesis (Baldassare 1999), and

cytoskeletal reorganization (Landry 1995). They control cell survival and adaptation by responding to chemical and physical stresses and have been shown in vitro to play an important role in osteoclastogenesis, but not yet in vivo (Asagiri 2006). They are all highly conserved serine-threonine kinases that are activated by upstream MAPK kinases through a Thr-Xxx-Tyr phosphorylation motif (Martin-Blanco 2000). Studies have shown that growth factors, mitogenic stimuli and tumor promoters in general, activate the ERK ½ pathway (Cobb 1999). The p38 and SAP/JNK pathways are stimulated by environmental stress and inflammatory cytokines (Zu 1998). The activation of the numerous transcription factors which function to stimulate the synthesis of various inflammatory proteins, including the cytokines TNF-α, IL-1β, and IL-6, is through phosphorylation from MAPKs. The activation of the MAPKs by LPS is initiated by a surprising number of pathways and likely reflects the different cell types used in the various studies (primary cells or cell lines, human or mouse cells, peritoneal or alveolar macrophages, etc.) (Schorey 2003). They are also involved in the activation of components of AP-1, thereby possibly modulating its activity during osteoclatogenesis. However, the molecular mechanisms as to how they function are not understood well (Chang 2001). Some of them have been shown to be activated downstream of RANK. It has been suggested that p38 is involved in osteoclast formation due to the effects of p38 inhibitor placed on RAW264.7 cells (Matsumoto 2000) and it has been shown to be involved with the induction of the cathepsin K gene (Matsumoto 2004). RANKL also activated the ERK1/2 kinases; however, osteoclastogenesis is potentiated when ERK activity is inhibited with a selective MEK inhibitor, PD98059, which suggests that the ERK pathway negatively

regulates osteoclastogenesis (Hotokezaka 2002). PD98059 is a potent and selective cell permeable inhibitor of MAP kinase. It blocks the activation of MEK, thereby inhibiting the phosphorylation and the activation of MAP kinase and is invaluable in helping elucidating the role of the MAPK cascade in a variety of biological systems. It has also been suggested that ERK is involved in osteoclast survival (Miyazaki 2000). Studies have also shown that MEK inhibitors cause a decrease of phosphorylated ERK and either suppress, or do not affect differentiation into osteoclasts from bone marrow cells (Lee 2002)

The p38 MAPK family is a group of 38 kDa intracellular signal transduction proteins which regulate gene expression in response to various extracellular stimuli like tumor necoris factor-α and interleukin-1 (Han 1994)and are predominantly activated through phosphorylation by upstream MAPK kinase 6 MKK6 (Boyle 2003). P38 is phosphorylated in response to RANKL, IL-1, TNF-α and LPS in bone marrow macrophages (Matsumoto 2000) which results in the downstream activation of the transcriptional regulator mi/Mitf, which controls the expression of the genes encoding TRAP and Cathepsin K, which are required by the mature osteoclast (Boyle 2003). Their physiological functions have been investigated through the use of specific inhibitors such as SB202190, a pyridinyl imidazole, which competes with ATP for the same binding site on the p38 kinase (Nemoto 1998); therefore, inhibiting the differentiation of bone marrow cells into osteoclasts by interfering with the RANKL-induced p38 MAP kinase activity (Matsumoto 2000).



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Macrophage Colony Stimulating Factor (M-CSF)

Entrance of mononuclear cells into the early preosteoclast pathway, after commitment to the osteoclast lineage, is in response to macrophage colony-stimulating factor (M-CSF). Proliferation, differentiation and survival of hematopoietic cells in the monocytic lineage require M-CSF (Suda 1992). Mesenchymal cells such as fibroblasts, osteoblasts and endothelial cells, as well as activated macrophages, synthesize this growth factor (Felix 1994). There is a macrophage deficiency and a lack of osteoclasts with the absence of a functional M-CSF in the op/op mouse (Kodama 1993). An injection of M-CSF can restore these deficiencies (Kodama 1993). Experimental evidence shows that M-CSF is not essential for the early stages of osteoclast development because the differentiation

and proliferation of osteoclast progenitors from inoculated stem cells can be supported by op/op marrow stromal cells (Lee 1994).

A variety of intracellular signals are activated which recruit adapter proteins and cytosolic kinases when M-CSF binds to its receptor, c-Fms. The cytoplasmic tail of c-Fms contain tyrosines 559 and 807 which play distinct roles in osteoclast differentiation and function (Zhao 2007). M-CSF receptor expression changes modulate the final lineage selection of the pluripotent monoblastic progenitor (Fan 1997). A direct respone to RANKL and interleukins require M-CSF induced genes. RANK and other RANK/NFκβ pathway components have been shown to be induced by M-CSF, thus providing a molecular explanation for the synergy of M-CSF and RANKL (Cappellen 2002) M-CSF has also been shown to induce interleukins, interferons, and their receptors (Capellen 2002). Inhibition of apoptosis of osteoclast precursors is also mediated by M-CSF (Woo 2002).

Activator protein-1 (AP-1)

AP-1 is a transcription factor comprised of a variety of dimers of members of the Fos, Jun and ATF families of proteins (Angel 1991). Ligands binding to cell-surface receptors, RANK for osteoclasts and Toll-like receptors (TLRs) for mononuclear phagocytes, lead to differentiation of the common precursors into either bone or immune lineages through expression of specific target genes that contain AP-1 binding sites (Zhao 2007). NF-κβ and AP-1 are both activated by RANK and TLRs. C-Fos and Fra-1 are members of this family, and c-Fos inactivation in mice results in arrested osteoclastogenesis and elevated numbers of marrow macrophages (Grigoriadis 1994). The introduction of c-Fos protein can rescue osteoclast

differentiation (Wang 1992). Therefore, c-Fos is essential for the commitment of hematopoietic precursors to become osteoclasts instead of mature macrophages (Tietelbaum 2000).

A redundant role is played by the Jun family of proteins, partners of the Fos family of proteins, in osteoclastogenesis. In knock-out mice, a deficiency in JunB or c-Jun leads to a sizable decrease in osteoclast formation but not a complete blockade, suggesting that during osteoclastogenesis, members of the Jun family substitute for each other (Arai 1999). AP-1 has a critical role in osteoclast differentiation and is also involved in numerous cell processes including: cell differentiation, proliferation, apoptosis and oncogenic transformation. Its activity is regulated through interactions with transcriptional and post-transcriptional regulators and by upstream kinases that link it to different signal transduction pathways (Jochum 2001). Since it is at the end of several signal transduction cascades, it is considered a stress responsive transcription factor complex (Masuo 2003).

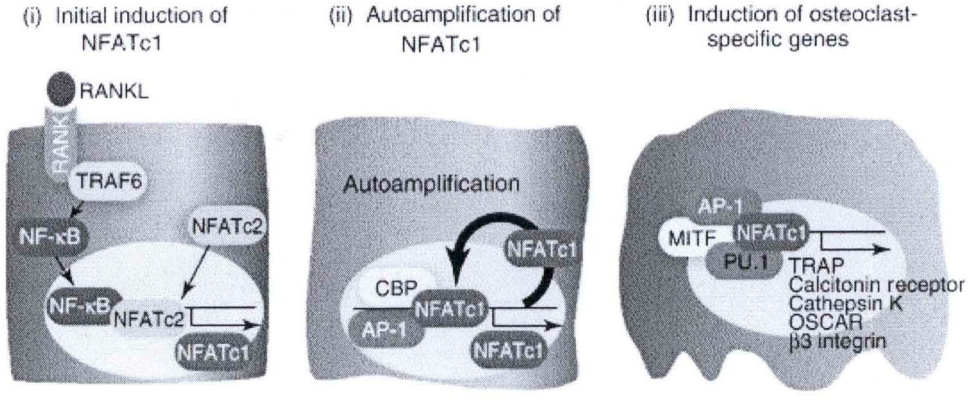
Nuclear factor of activated T-cell cytoplasmic (NFATc)

Nuclear factor of activated T-cell cytoplasmic (NFATc1) was shown to be strongly induced by RANKL in a genome-wide search for the RANKL-inducible genes required specifically for the terminal differentiation of osteoclasts (Ishida 2002). There are four members of this transcription factor family (NFATc1 through NFATc4) which were originally identified in T-cells (Zhao 2007). NFATc1 has been referred to as the master transcription factor for osteoclastogenesis (Kim 2005). The immune, cardiovascular and skeletal systems are all regulated by them to some degree (Crabtree 2002). Calcineurin, a specific phosphatase that is activated by

calcium/calmodulin signaling, mediates the activation of the NFAT family. NFATc1 is controlled by the phosphatase calcineurin, which plays a critical role in the coupling of calcium signals with cellular responses (Klee 1998). Once activated by an increase of the intracellular calcium concentration, calcineurin induces the translocation of NFATc1 into the nucleus where it activates the transcription of specific target genes (Kim 2005). RANKL-induced osteoclastogenesis and NFATc1 induction are strongly inhibited by the calcineruin inhibitor FK506. This shows the importance of calcium signaling during osteoclast differentiation (Takayanagi 2002). FK506 inhibits immune function by blocking the enzyme activity of calcineurin (Fukunaga 2004). Complexes are formed when the drug binds with FK506-binding protein (FKBP) which in turn reduces the expression of cytokines (Liu 1991).

It has been suggested that NFATc1 is one of the key target genes of NF-κβ in the early phase of osteoclastogenesis since its induction was impaired in TRAF6 negative cells (Takayanagi 2002), through the use of an NF-κβ inhibitor (Takatsuma 2005) and in p50/p52 deficent cells (Li 2004). It has also been shown that NF-κβ is recruited to the NFATc1 promoter immediately after RANKL stimulation (Asagiri 20056). NFATc2 is another preexisting molecule that is recruited to the NFATc1 promoter within minutes of RANKL stimulation and at the same time as NF-κB (Asagiri 2006). However, the loss of NFATc2 might be compensated for by other NFATs, since NFATc2 deficient mice have no defects in osteoclastogenesis ((Asagiri 2005). Promoter analyses shows that NFATc1 regulates a number of osteoclast-specific genes such as TRAP (Kim 2005), calcitonin receptor (Anusaksathien 2001), cathepsin K (Kim 2005) and β3 integrins (Crotti 2006); however, the critical binding sites have

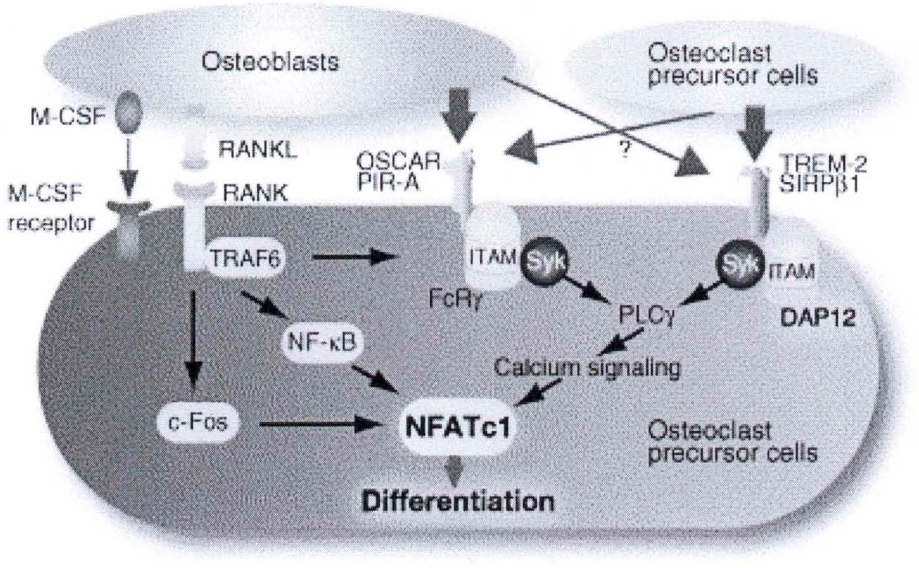
not been identified fully. It is not understood how the target genes of NFATc1 promote the differentiation process (Asagiri 2006). In the context of transcriptional control by NFATc1, the process of osteoclast differentiation can be divided into three stages. First, the recruitment of TRAF6 due to RANK-RANKL interaction leads to the activation of downstream molecules such as NF-κβ. The *NFATc1* promoter recruits NFATc2 in the early phase also. The initiation of *NFATc1* occurs through the cooperation of NFκβ and NFATc2. Secondly, NFATc1, stimulated by calcium signaling, is activated and binds to its own promoter which leads to the autoamplification of *NFATc1*. AP-1 containing c-fos is essential for this to occur. There is selective recruitment of NFATc1, induced by RANKL, to the promoter of *NFATc1*, but not to that of *NFATc2*. Lastly, a transcriptional complex containing NFATc1 and cooperators such as as AP-1, PU.1 and micropthalmia-associated transcription factor (MITF) activate a number of osteoclast-specific genes such as *cathepsin K, TRAP, calcitonin receptor and OSCAR* (Asagiri 2006).



Asagiri, Bone 2006.

Costimulatory signals for RANK

NFATc1 also regulates the osteoclast-specific immunoreceptor osteoclast-associated receptor (OSCAR) (Kim 2005). OSCAR is an immunoglobulin-like receptor expressed by osteoclasts. It is involved in the cell to cell interaction between osteoblasts and osteoclasts (Kim 2002) and has been shown to associate with an adaptor molecule, FcRγ (Koga 2004) which contains an immunoreceptor tyrosine-based activation motif (ITAM), that is essential for the activation of calcium signaling in immune cells (Reth 1989). DAP12 is another ITAM-harboring adaptor, and has been shown to have involvement in the formation and function of osteoclasts (Kaifu 2003). Due to the differentiation blockade of osteoclasts, mice deficient in FcRy and DAP12 exhibit severe osteopetrosis, demonstrating that immunoglobulin-like receptors, which include OSCAR, triggering receptor expressed in myeloid cells (TREM)-2, signal-regulatory protein (SIRP) Beta1, and paired immunoglobulin-like receptor (PIR)-A associated with FcRy and DAP12 are essential for osteoclastogenesis (Koga 2004). However, the precise function and ligands of these receptors is not known. ITAM-mediated signals, costimulatory signals for RANK, work with RANK to stimulate calcium signaling through ITAM phosphorylation resulting in the activation of Syk family kinase and phospholipase Cγ (PLCγ). The immunoglobulin-like receptors associated with FcRy and DAP12 have been identified as essential RANK partners during osteoclastogenesis (Baron 2004), however, how RANK can specifically induce osteoclastogenesis in cooperation with ITAM signaling, is not fully understood (Asagiri 2006).



Asagiri, et al. Bone (2006)

Micropthalmia-associated transcription factor (Mtif)

The basic/helix-loop-helix/leucine zipper (b-HLH-ZIP) transcription factor subfamily has microphthalmia-associated transcription factor (Mitf), Tfe3, Tfeb, and Tfec as members (Zhao 2007). The proper development of osteoclasts, melanocytes, retinal pigment epithelial cells, mast cells and natural killer cells require Mitf. Defective osteoclast development with osteopetrosis is found with Mitf mutations (Hershey 2004). There is induction of the osteoclast-specific marker of the TRAP gene when MITF interacts with either PU.1 or PU.1 interacting protein (Partington 2004). The *OSCAR* gene is activated by PU.1 and MITF transcription factors also. RANKL targets MITF in the osteoclast signaling pathway, and MITF phosphorylation causes an increase in OC-specific gene expression (Mansky 2002).

Myc

There are four related genes in the Myc family which are are key regulators of cell proliferation and contribute to the genesis of many human tumors: c-Myc, N-Myc, L-Myc, and S-Myc (Adhikary 2005). RANKL-induced osteoclast-like cells show

upregualtion of c-Myc, with absence of c-Myc in undifferentiated cells (Zhao 2007). RANKL-induced osteoclast formation was blocked in dominant negative Myc in raw 264.7 cells; therefore, expression of c-Myc is required for RANKL-induced osteoclastogenesis and is a downstream target of RANKL (Battaglino 2002).

Src

Deletion of the gene encoding c-Src, which is a member of a family of nine NRTKs that associate with the cytoplasmic surface of cellular membranes, results in osteopetrosis, and is a result of the inablility of the mature osteoclast to resorb bone (Brown 1996).

Tumor Necrosis Factor (TNF)

In the eighteenth century, shrinkage of tumors in some cancer patients who were exposed to severe bacterial infections, led to the belief that some biological factor mediated this tumor necrotic activity (Coley 1893). In 1968, lymphotoxin, a soluble protein produced by T-lymphocytes was discovered (Granger 1968). Experiments demonstrated in 1975 that subsequent to stimulation of their reticuloendothelial system and lipopolysaccharide challenge, a protein, termed "Tumor Necrosis Factor" (TNF), was released into the circulation of animals (Carswell 1975). It was named as such because this protein was shown to cause rapid necrotic regression of certain forms of tumors. However, not all tumors were susceptible to it and any therapeutic potential was diminished by its systemic toxicity at effective concentrations (Idriss 2000).

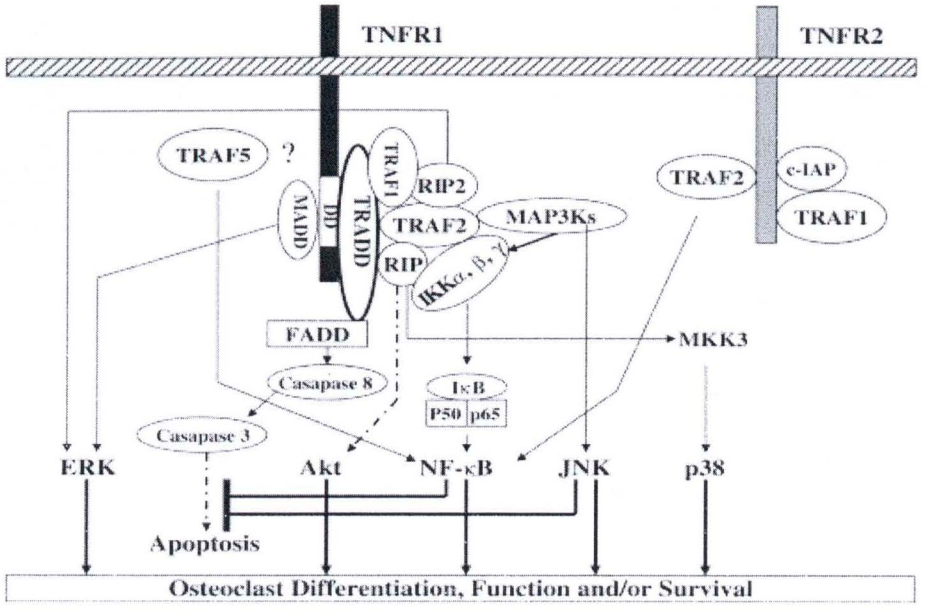
TNF-α and lymphotoxin (TNFbeta and LT-alpha) both define two structurally and functionally related proteins (Aggarwal 1991). Since their isolation and cloning in

1984 from activated macrophages and T cells, (Gray 1984), the TNF family has been expanded to include nineteen members (Locksley 2001). TNF-α has been established as a major mediator of inflammation; however, other family members regulate a variety of cellular functions such as cell differentiation, function, survival and apoptosis (Gaur 2003).

TNF Receptors

TNF receptors (TNFR) are found on almost all cell types except for erythrocytes and unstimulated T lymphocytes. There are twenty-nine known members which are usually type I transmembrane proteins; however, a few TNFR members are secreted soluble proteins. (Feng 2005). No correlation has been found between receptor density and the magnitude or direction of the TNF-induced response (Beyaert).

Many membrane bound receptors, including the TNF-α receptors,TNFR1 and TNFR2, form what is known as the TNF receptor superfamily (Bazan 1993). Since they lack enzyme activity in their intracellular domains, TNFR family members transduce signaling through the recruitment of adapter proteins, primarily death domain-containing proteins and members of the TRAF family.



Feng, Genes, 2005

TNF-α and its receptors TNFR1 and TNFR2

TNF-α is a cytokine that promotes the inflammatory response. It is mainly produced by macrophages but also by endothelial cells, fibroblasts, mast cells and neuronal tissue. TNF-α has varied biological functions with a somewhat complex mechanism of action. Its intracellular signaling pathways have been delineated and its target genes that control its function have been identified (Beutler 1999). It confers resistance to certain infections but causes pathological complications in other instances (Fiers 1991). Lack of resistance to leishmania and failure to form splenic germinal centres were shown in mutant phenotype of knock-out mice that lacked the gene for TNF-α (Goldfield 1996). The important role of TNF-α in conferring immunity and a proper inflammatory response was shown with TNFR knockout mice that, when challenged with bacterial endotoxins, displayed an increased susceptibility to microbial infection and a suppressed inflammatory response (Acton 1996).

Immunostimulation, resistance to infection agents, resistance to tumors (Aggarwal 1991), sleep regulation (Krueger 1998) and embryonic development (Wride 1995) are amongst the processes that TNF-α plays a role in. However, due to TNF-α circulation, parasitic, bacterial and viral infections become more pathogenic or fatal (Fiers 1991).

The TNF-α gene is a single copy gene on the short arm of human chromosome 6 and murine chromosome 17 and is preceded by TNF-β (Spriggs 1992). TNF-α exists in a membrane bound an a soluble form, with each possibly carrying out a distinct physiological role (Beyaert1994). It is first produced as type II membrane protein, unlike TNF-β which is secreted (Idriss 2000) and exists in a more diverse range of cells than TNF-β. It is produced in a variety of hematopoietic and non-hematopoietic cells, both normal and malignant which include macrophages, CD4+ and CD8+ T-lymphocytes, B-lymphocytes, NK cells, neutrophils, astrocytes, endothelial cells, smooth muscle cells and tumor cell lines of non-hematopoietic origin (Vilcek 1991).

TNF-α exerts its function via two receptors, TNFR1 (also known as p55), which contains a death domain (Himmler 1990) and TNFR2 (also known as p75) which lacks a death domain (Gray 1990). TNFR-1 is widely distributed on cells such as fibroblasts and epithelial cells, and TNFR-2 is mainly confined to cells of hematopoietic origin (Armitage 1994). As discussed earlier, RANKL and RANK are essential for osteoclastogenesis since the mice deficient for either RANKL or RANK completely lack osteoclasts (Li 2000). In contrast, the mice lacking TNF-α or its receptors do not exhibit any bone defects (Marino 1997) Both TNFR1 and TNFR2 are implicated in osteoclast formation and function. In pathological

circumstances,TNFR1 positively regulates osteoclast formation and function (Abu-Amer 2000), in contrast, TNFR2 exerts an inhibitory effect on osteoclast formation and function (Abu-Amer 2000). Signaling pathways activated by TNFR1 and TNFR2 have been intensively investigated in a variety of other cell types. TNFR1 receptor binding leads to activation of factors such as capase 8 and apoptosis. The second group which includes TNFR2, CD40 or CD30 bind TRAFS, which are molecular adapters that link these surface receptors to downstream signaling which activate JNK, SAPK and NFαβ (Locksley 2001). It has been shown that TRAF binding domains, particularly TRAF6, are functionally important for the RANK-dependent induction of NF-κβ and c-Jun NH2-terminal kinase (JNK) activities. When the TRAF6 interaction domain was deleted, NF-κβ signaling mediated by RANK was completely inhibited (Darnay 1998). However, the precise signaling cascades leading to the activation of these pathways have not been functionally established in osteoclast precursor/osteoclasts and are based on studies involving cells other than osteoclast precursors/osteoclasts (Feng 2005). Further studies are needed to evaluate whether these signaling cascades are used to activate NF-κβ, JNK, p38, ERK, and Akt in osteoclast precursors/osteoclasts (Feng 2005).

It has also been shown that TNF-α can modulate osteoclast formation and function by enhancing the expression of RANKL by osteoblasts and stromal cells (Hofbauer 1999) and is mediated via TNFR1 (Abu-Amer 2004). However, the molecular mechanism by which TNF-α regulates RANKL gene expression in osteoblasts/stromal cells is still not known.

TNF-α also stimulates M-CSF production by murine or human stromal cells (Srivastava 2001). TNF-α is among the most potent of the osteoclastogenic cytokines produced during inflammation. Not only has it been implicated in the pathogenesis of conditions which include rheumatoid arthritis, orthopedic implant loosening, and other forms of chronic inflammatory osteolysis (Birkedal-Hansen 1993), it also mediates LPS-stimulated osteoclastogenesis via its p55 receptor, through a mechanism involving activation of NF-κB (Abu-Amer 1997). A topic of considerable debate has been whether TNF-α promotes osteoclast formation independently of RANK signaling. It has been demonstrated by two independent groups that TNF-α promotes osteoclast formation in vitro despite RANK signaling blockade (Azuma 2000, Kobayashi 2000); however, others report that "permissive" levels of RANKL are required for TNF-alpha-induced osteoclastogenesis (Lam 2000).

The purpose of this study was four-fold: to determine if LPS shares the same pathway with RANK for osteoclastogenesis, to determine whether TNF-α can stimulate osteoclast formation directly, to examine the effect of LPS on TNF-α release during osteoclast formation and to examine the effect of blocking the TNF-α receptor.

Material and Methods

Reagents

All media components were purchased from GIBCO. RANKL and TNFα, TNF-α ELISA was obtained from R & D Systems. *E. Coli* LPS was obtained from Sigma. SB202190, PD98059, LY294002 and FK506 were obtained from Calbiochem.

Preparation of osteoclasts

Osteoclast-like cells (OCL) were differentiated from RAW 264.7 cells, a mouse hematopoietic cell line (American Type Culture collection, Rockville, MD). For morphological examination, RAW 264.7 cells were plated at a density of 40,000 cells/well in 24 well plates in Alpha modified Eagle medium with 10% fetal bovine serum (Invitrogen, Carlsbad, California). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

TRAP Assay

Tartrate resistant acid phosphatase (TRAP) is a marker enzyme specific for osteoclasts. At 96 h of culture, cells were fixed with 2% paraformaldehyde, washed with phosphate buffered saline, and treated for 20 minutes with 0.2% Triton X-100 solution to permeabilize cell membranes. Cytochemical staining of tartrate-resistant acid phosphatase (TRAP)-positive cells was performed. TRAP-positive cells appeared dark red. Only TRAP-positive cells with more than 3 nuclei were counted. The values are expressed as means ± SE of triplicate cultures.

ELISA

Concentrations of TNFα in culture supernatants were determined by ELISA in triplicate with commercial ELISA Duo systems (R&D systems), according to the respective manufactures' instructions. For each sample and assay, the mean of the triplicate measurement were calculated.

Statistical Analysis

Data were expressed as mean values ± SD. Statistical significance of differences was determined by one way ANOVA and followed by post hoc test (Fisher's protected least significant difference). Differences were considered statistically significant at $p<0.05$.

Results

Each group contained three wells and was run in triplicate. Three investigators counted the cells.

LPS/RANKL Pathway

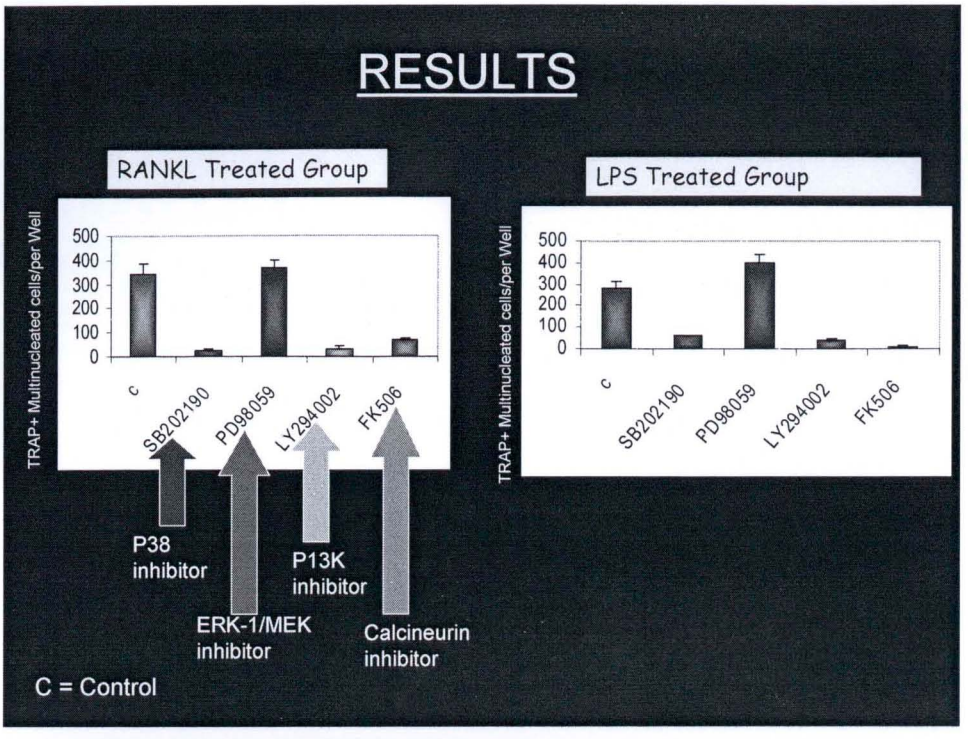
In order to investigate if LPS and RANKL share a common pathway for activation of osteoclastogenesis, various receptor or pathway inhibitors were used. In the graph below, the upper portion represents the RANKL treated group and the lower portion represents the LPS treated group. When cells treated with RANKL were cultured with OPG, which is a soluble inhibitory peptide that binds to RANKL, thereby reducing its availability and blocking RANK signaling, there was almost complete inhibition of osteoclast formation. However, when this group was cultured with an antibody to the LPS receptor, TLR4, no effect is seen. Conversely, when the LPS group was cultured with OPG, there was osteoclast formation, however, with use of anti-TLR4, there is a reduction in osteoclast formation.

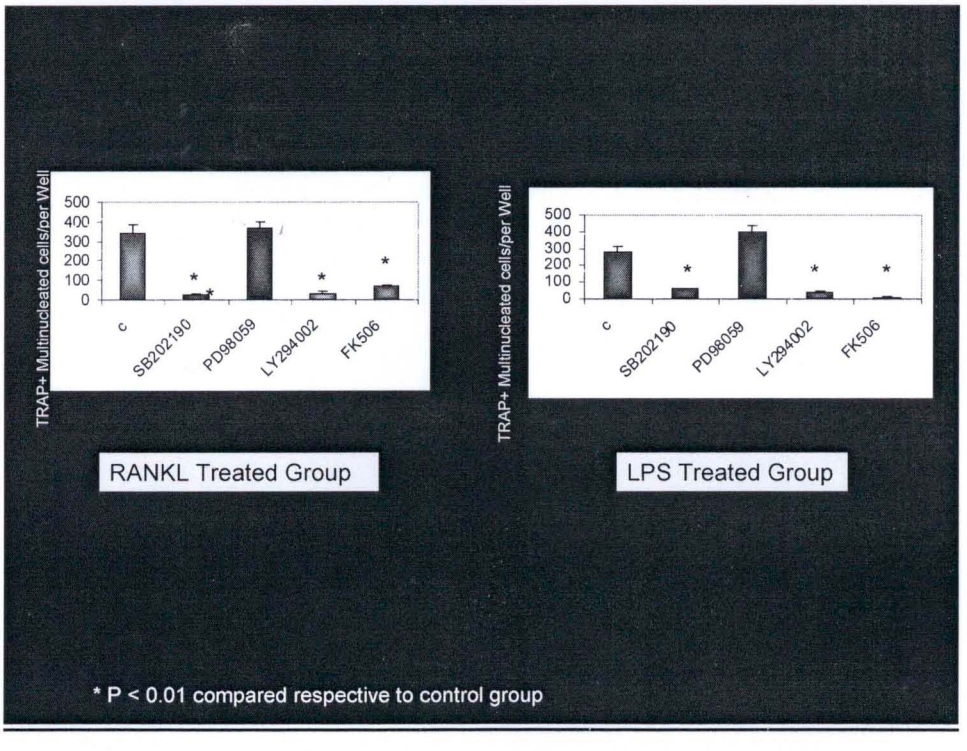
RANKL – 325 osteoclasts LPS – 225 osteoclasts

RANKL + OPG – 56 osteoclasts LPS + OPG – 243 osteoclasts

RANKL + Anti-TLR4 – 301 osteoclasts LPS + Anti-TLR4 – 85 osteoclasts

As seen in the following graphs, there were dramatic reductions in osteoclast formation in the LPS and RANKL group when pathway inhibitors of p38 MAPK, PI3K or calcineurin/NFAT pathways were used; however, there was no inhibition when an inhibitor for the MEKK pathway was used.

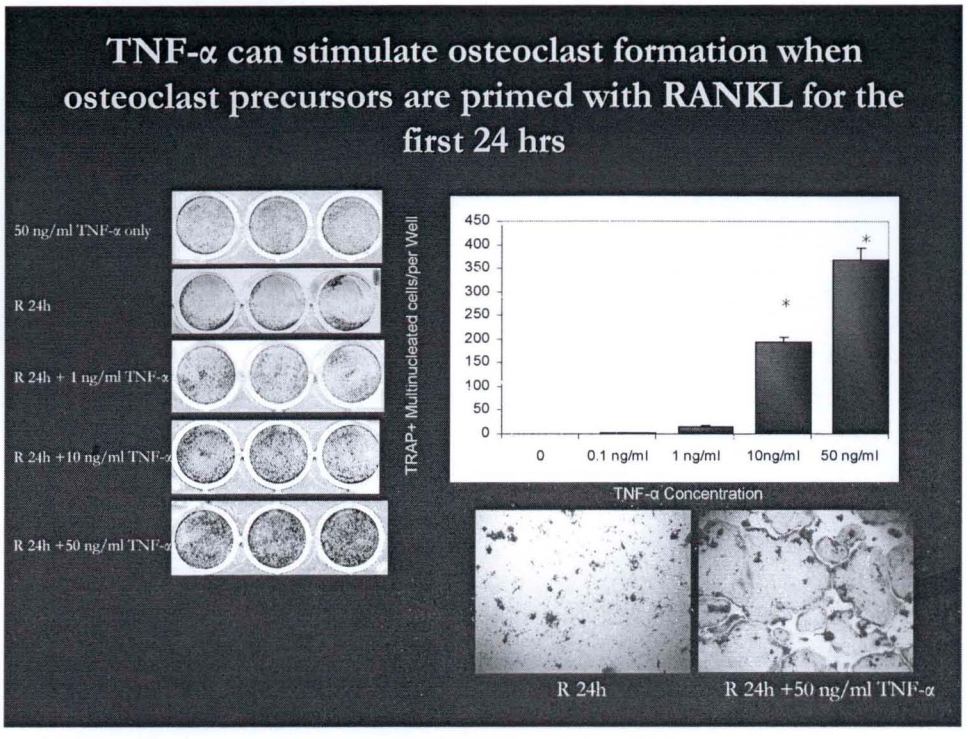

RESULTS
RANKL Treated Group
LPS Treated Group
TRAP+ Multinucleated cells/per Well
500
400
300
200
100
0
c
SB202190
PD98059
LY294002
FK506
P38 inhibitor
P13K inhibitor
ERK-1/MEK inhibitor
Calcineurin inhibitor
C = Control


TRAP+ Multinucleated cells/per Well
500
400
300
200
100
0
c
SB202190
PD98059
LY294002
FK506
RANKL Treated Group
LPS Treated Group
* P < 0.01 compared respective to control group

RANKL treated group	LPS Treated group
Control – 285 osteoclasts	320 osteoclasts
MAPK inhibitor SB202190 – 62 osteoclasts	84 osteoclasts
MEKK inhibitor PD98059 – 395 osteoclasts	440 osteoclasts
P13K inhibitor LY294002 – 40 osteoclasts	64 osteoclasts
NFAT2/Calcineurin inhibito FK506 – 10 osteoclasts	20 osteoclasts

TNF-α stimulation of osteoclastogenesis

Osteoclast precursors were pretreated with RANKL for 24 hours. RANKL was removed and TNF-α was supplemented at doses of 0.1 ng/ml, 10 ng/ml and 50 ng/ml for an additional 72 hours. In addition, two sets of cell groups were treated with 50 ng/ml of TNF only or RANKL only for 24 hours. The cells that were treated with only TNF-α or RANKL for only 24 hours had no osteoclast formation. With 10 ng/ml and 50 ng/ml of TNF-α, as shown in the graph below, there was marked formation of TRAP+ multinucleated cells in the culture. This indicates that TNF-α dose-dependently stimulates osteoclast formation if previously exposed to RANKL.



LPS-stimulated release of TNF-alpha

The effect of LPS on TNF-α release during osteoclast formation was examined by ELISA.

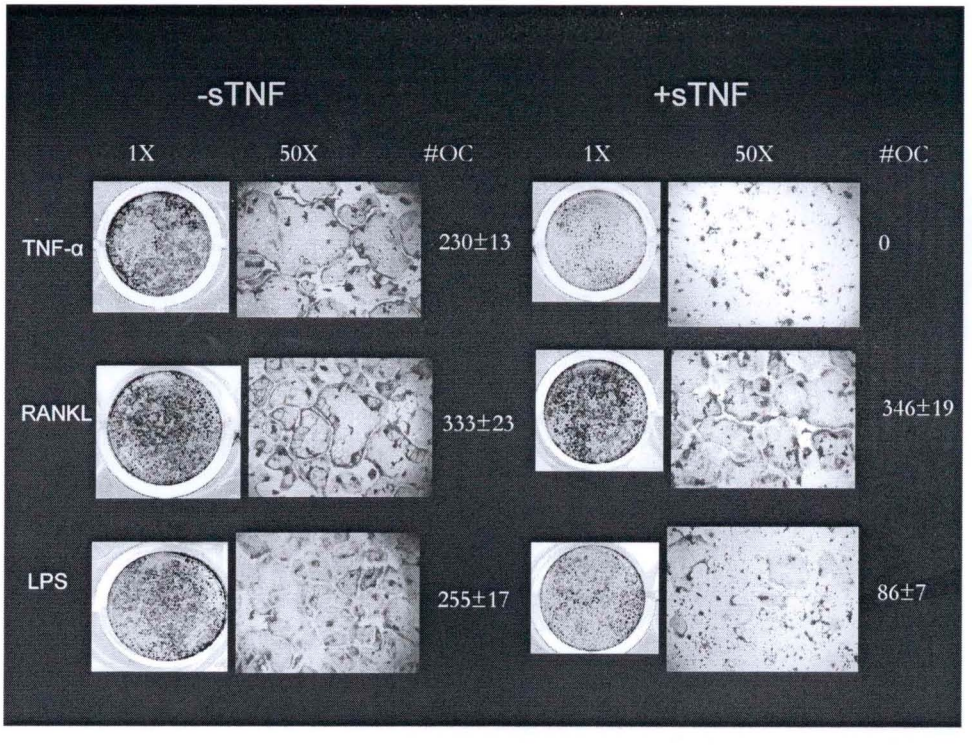
- ELISA results

Group	Treatment	TNF-α Release
A	No treatment	Not detectable
B	100 ng/ml LPS	23.49± 0.98 ng/ml
C	50 ng/ml RANKL (24 h)+ 100 ng/ml LPS (72h)	4.32 ± 0.12 ng/ml

Group A cells which received no treatment, had no TNF-α release detected by ELISA. Group B cells which received 100 ng/ml of LPS had a TNF-α release of 23.49 ng/ml detected. Group C cells which received 50 ng/ml of RANKL which was then removed, and then were treated with 100 ng/ml of LPS, had TNF-α release of 4.32 ng/ml detected by ELISA.

Blocking the TNF-α receptor

The effect of blocking the TNF-α receptor was examined.. Below is a summary of this treatment.



On the left panel above are the cells that received RANKL for the first 24 hours and were then treated with TNF-α, additional RANKL or LPS. On the right panel are the cells that were treated with the TNF-α blocker. Osteoclast formation due to TNF-α

stimulation was completely abolished. Osteoclast formation due to RANKL was unaffected, and osteoclast formation due to LPS decreased by 60%.

Discussion

Chronic inflammation of bone or periosseous tissues by Gram-negative bacteria as seen in endodontic infection causes osteolysis due to osteoclastic bone resorption, a process driven by host inflammatory responses. There is also a causative role of lipopolysaccharide-producing bacteria in the pathogenesis of apical periodontitis (Sundqvist 1976). Periapical bone destruction and apical periodontitis occur when the pulp is infected with certain anaerobic Gram-negative species since the necrotic pulp is insufficient by itself to cause these conditions (Sundqvist 1976). Prolonged or excessive release of cytokines plays a central role in the pathogenesis of inflammatory bone destruction. One of the most potent osteoclastogenic cytokines produced in inflammation is TNF-α (Lam 2000). It has been shown to be involved in the pathogenesis of conditions including rheumatoid arthritis, orthopedic implant loosening and other forms of chronic inflammatory osteolysis (Nair 1996). Lipopolysaccharide-stimulated osteoclastogenesis via its p55 (TNFR1) receptor which involves activation of NF-kβ is mediated by TNF-α (Abu-Amer 1997).

The host defense response to LPS includes expression of a large variety of pro-inflammatory cytokines which include TNF-α, interleukin-1β, IL-6, interferon gamma, IL-12, IFN inducible protein-10, chemotactic cytokines, like monocyte chemotactic protein-5, IL-8, MIP-1alpha and MIP-2,and prostaglandin E_2 (Madianos 2005). The mechanism of action of LPS is not fully understood; however, it stimulates a variety of mechanisms during inflammation. LPS induces RANKL

expression in osteoblasts, activated T-cells, synovial fibroblasts and bone marrow stromal cells. It has been shown that LPS is able to induce osteoclast formation after RAW 264.7 cells were primed with RANKL (Jiang 2003). LPS can also induce the activation of NF-kB and upregulate the expression levels of TNF-alpha (Jiang 2003). It is known that LPS primes polymorphonuclear leukocytes (PMNs) to release reactive oxygen radicals and causes an increase in the surface expression and activity of neutrophil adhesion molecules (Lynn 1992). It is also known that the adherence of PMN's and monocytes directly to endothelium is promoted by LPS (Lynn 1992). It has also been shown that after three to seven months of experimentally applied bacterial LPS into the root canals of monkeys, the periapical tissues showed signs of resorptive bone loss inflammatory reactions (Dahlen 1981). This provides strong evidence that LPS is essential to the formation of periapical lesions.

The first host protein involved in LPS recognition is LPS-binding protein, which complexes with the lipid A moiety of LPS. This complex allows LPS to dock at the LPS receptor complex by forming a complex with CD14. This allows LPS to be transferred to the LPS receptor complex composed of TLR4 and MD-2 (Palsson-McDermott 2004). It was initially thought that CD14 was the long sought after receptor for LPS because antibodies to CD14 blocked binding of LPS/LPB; however, because CD14 anchors without a transmembrane domain, it was also unlikely that CD14 alone could activate a signal in response to LPS (Wright 1990). It has been shown through subsequent studies that TLR4 is the actual LPS receptor (Poltorak 1998). The role of CD14 in TLR4 signaling is that it binds LPS and presents it to MD-2 and TLR4. It has also been shown that CD14 is important in TLR2 signaling.

It acts as a membrane receptor for bacterial products other than LPS, such as peptidoglycan and lipoarabinomannan, presenting these to TLR2 (Muroi 2002).

The receptor for LPS has been established as TLR4; however, TLR4 also recognizes lipotechoic acid (LTA), fibronectin, the fusion protein of respiratory synctial virus (RSV) and taxol, a plant diterpene structurally unrelated to LPS but it exhibits LPS-mimetic effects on murine cells (Poltorak 1998, Hoshino 1999, Okamura 2001, Kurt-Jones 2000, Kawasaki 2000). To further complicate LPS signaling, LPS derived from different strains of bacteria appear to be recognized by different clusters of receptors, therefore, giving rise to different cellular responses (Hirschfield 2001). It has been suggested in one study that the shape of the LPS molecule is critical for a cellular response to LPS and that only LPS with a conically shaped lipid A portion will act as a ligand for TLR4 (Netea 2002). Lipid A portions of LPS that are cylindrically shaped, as found in several non-enterobacteria, as well as precursors and analogues of toxic lipid A from E.coli LPS will bind and activate TLR2 and may even act as antagonists to TLR4 (Triantafilou 2004). It has also been demonstrated that less recruitment of TLR4/MD-2 within lipid rafts leads to activation of the mitogen-activated protein kinase (MAPK) pathways without activating NF-kB; therefore, the structure of LPS is essential for the formation of specific TLR receptor clusters in response to diverse bacterial products (Triantafilou 2004).

LPS not only causes MD-2/TLR4 to homodimerize, it also causes several other combinations of signalling proteins to become associated with the receptor complex which is determined by the form of LPS bound to MD-2/TLR4. Heat shock proteins

90 and 70, CD55, CD11/CD18 have been shown as components of the TLR signaling complex, although, as of yet, the importance of these signaling proteins remains uncertain (Triantafilou 2001, Byrd 1999, Heine 2003).

The LPS signaling pathways studied most extensively are those activated by TLR4 that make use of adapter proteins: MyD88, Mal, TRIF, and TRAM. Evidence is emerging that shows the TLR4-mediated response to LPS can be divided into two categories: an early MyD88-dependent response; and a delayed MyD88-independent response (Palsson-McDermott 2004).

TLR4 is unique because it acts through both the dependent and independent pathways, and it has been suggested that the potent inflammatory effects of LPS may be due to the synergy between these two pathways (Akira 2004). The dependent pathway uses the adapter molecule MyD88 and leads to early activation of NF-κβ and production of cytokines such as TNF-α (Akira 2004). The independent pathway signals through Toll-IL-1R domain-containing adaptor-inducing IFN-β leading to IFN-β production (Yamamoto 2002) It also, in a delayed fashion, activates through NF-κβ, leading to production of inflammatory cytokines (Covert 2005).

It has been observed that pre-treatment with agonists of the dependent pathway prime inflammatory cytokines and IFN-β responses to subsequent LPS treatment, and conversely, cells are tolerized to these agonists when pretreated with LPS (Bagchi 2007). The hypothesis that inhibition of proximal pathways and augmentation of distal pathway activity may contribute to tolerance and priming is supported with this type of response pattern. Therefore, LPS and other dependent pathway agonists may inhibit the proximal dependent path and increase the activity of distal components of

both the independent and dependent pathways (Bagchi 2007). The decreased response to a dependent-specific agonist following exposure to LPS would result from the signal not getting through the proximal dependent pathway. However, when cells are treated first with the agonist, despite proximal inhibition of the dependent pathway, LPS can still access the up-regulated distal pathways via the proximal, non-inhibited independent pathway (Bagchi 2007). The interplay between and the modulation of inflammatory effects of TLRs is highly complex, and studies indicate that patterns of inflammatory responses to combinations of TLR agonists can be predicted based on utilization of the dependent and independent pathways (Bagchi 2007). The dependent and independent pathways share some distal intermediaries, such as TRAF-6 and NF-κβ (Jiang 2004). However, highly purified TLR agonists were used, so it is difficult to directly compare these responses with the more complicated environment that exists in infection (Bagchi 2007).

MyD88-dependent signaling

MyD88 is composed of a C-terminal TIR domain separated from an N-terminal death domain (DD) (Burns 1998). Protein-protein interactions through other DD sequences are mediated by this DD. This mechanism is utilized by many signaling complexes which induce responses such as cytoxicity, activation of MAPK and activation of transcription factors such as NF-κβ (Palsson-McDermott 2004). It was initially shown that MyD88 associated with the Type I Il-1R (IL-1RI) through its TIR domain, which was also then shown to occur for TLR4 (Wesche 1997). Studies using MyD88-/- mice revealed both MyD88-dependent and MyD88-independent pathways of TLR4 signalling.

In comparison to wild-type mice, NF-κβ and MAPK activation occurred but was delayed in MyD88 deficient mice and these mice were resistant to the lethal effects of LPS. The induction of IFN-β to LPS was also unaffected (Jiang 2003). The role of MyD88 as an early responder to LPS was proposed when MyD-88 deficient splenocytes were incapable of proliferation in response to LPS (Poltorak 1998).

Mal, a second adapter protein, was then described in TLR4 signaling (Fitzgerald 2001) and it was thought that it may be the adapter involved in the MyD88-independent pathway. Mal knockout mice studies however proved that Mal was an essential adapter that works together with MyD88 (Oshiumi 2003). Mal-deficient mice displayed a resistance to the toxic effects of LPS and did not produce TNF, IL-6 or IL-12p40 in response to LPS. These mice also showed a delayed activation of NF-κβ and MAPK in response to LPS, which strengthened the theory that Mal is essential for, and acts together with, MyD88 (Palsson-McDermott 2004). Mal has been shown to be differentially involved in signaling by different TLRs and may provide some of the specificity in the downstream events of the different receptors (Palsson-McDermott 2004). Horng (2002) and Yamamoto (2002) simultaneously showed that Mal-deficient mice had normal responses to TLR5, TLR7, TLR9, IL-1 and IL-18 which suggested that these receptors signal independently from Mal. However, these mice exhibit impaired responses, not just to LPS but also to the ligand for TLR2 (Horng 2002).

Downstream events in the activation of the MyD88-dependent pathway by LPS, leading the to the activation of NF-κβ and the MAPK pathways are shared with the IL-1 pathway which has been well-studied (Palsson-McDermott 2004). The

activation of NF-κβ, in a simplified model, starts with the association of IRAK-1 and IRAK-4 with the receptor complex. Autophosphorylation of IRAK-1 occurs in two substeps, giving rise to hyperphosphorylated IRAK-1 which casuse dissociation from the receptor complex and association of IRAK with Traf6 ((Cao 1996). TRAF6 then becomes activated and associated with TAB-2, which activates the MAPK kinase TAK1 (transforming growth factor-β-activated kinase) which is constitutively associated with its adapter protein, TAB-1 (Ninomiya-Tsuji 1999). At this point, TAK-1 acts as a common activator of NF-κβ, p-38 and c-jun N-terminal kinase (JNK) pathways (Palsson-McDermott 2004). The assembly of a high-molecular-weight protein complex known as a signalsome initiates the activation of NF-κβ. The inhibitory protein κβ, made up of IκB-α and IκB-β, along with a scaffolding protein named IKKγ (also known as NEMO) make up the signalsome (Palsso-McDermott 2004). Eventual phosphorylation of these inhibitory-binding proteins results in their ubiquitination and degradation, releasing NF-κβ which can then translocate into the nucleus (Palsson-McDermott 2004) to begin transcription.

MyD88-independent signaling

After both Mal and MyD88 had been shown to be responsible for conveying the early response to LPS, the adapter (s) for the delayed MyD88-independent pathway were still missing. TRIF was the next adapter to be identified (Yamomoto 2002) and it was shown to activate NF-κβ when over-expressed; however, not as potently as the activation of NF-κβ by MyD88 and Mal (Palsson-McDermott 2004). Important evidence for the role of TRIF in TLR4 signaling came when TRIF-deficient mice were used to demonstrate the involvement of TRIF in the MyD88-independent

pathway in response to LPS. The activation of NF-κβ in response to LPS in TRIF-/- mice was measured and almost normal, although when the cells were deficient in TRIF as well as MyD88, the NF-κβ response to LPS was totally abolished. TRIF, most importantly, was also shown to be the sole adapter used by TRL3 (Yamamoto 2003)

Even though these studies established TRIF as a key adapter in the MyD88-independent signaling pathway by TLR4, they also showed poor direct interaction beween TLR4 and TRIF; therefore, the question still remained as to what further adapter molecule was involved in this pathway. Two different groups independently identified TRAM as the adapter candidate (Fitzgerald 2003) (Oshiumi 2003). TRAM was shown to activate IRF-3, IRF-7 and NF-κβ independently of MyD88. It was also shown to be uniquely required by TLR4 in order to signal which was in contrast to TRIF that was also required by TLR3 (Fitzgerald 2003). When measuring the activation of NF-κβ and IFN-β promoter activity, TRAM, on its own, displays little or no signaling activity; therefore, it has been suggested that it has no other role than acting as a bridge between TLR4 and TRIF (Oshiumu 2003). It has also been shown that TRAM cannot interact with TLR2, TLR5, TLR6, TLR7, TLR8 or TLR9 and only weakly, or not at all with TLR3, further evidence for TLR4 specificity. Since they were defective in their response to LPS in a manner similar to that of the TRIF -/- mice, mice lacking functional TRAM showed that TRAM was essential for the MyD88-independent signaling pathway in response to LPS (Yamamoto 2003). The MyD88-dependent pathway appeared to function normally, whereas the MyD88-independent response to LPS was completely eliminated. The

MyD88-independent pathway is responsible for the later activation of NF-κβ and involves TRIF binding to TRAF6 with the ultimate translocation of NF-κβ to the nucleus after ubiquitination and degradation of Ikβ (Sato 2003).

The current simplified working model for an early MyD88- dependent and later MyD88 independent-mediated response to LPS is as follows: circulating LBP recognizes LPS in the plasma and brings it to CD14. With the help of CD14, LPS bind to MD-2 and TLR4. This induces homodimerization of TLR4 which then recruits MyD88 and Mal to the receptor complex (Palsson-McDermott 2004). LPS signaling leads to the early activation and phosphorylation of IRAK, TRAF6 becomes activated, which gives rise to the expression of numerous pro-inflammatory genes. As a later response to LPS, TLR4 gives rise to the activation of TRAF6 and TBK1, an event mediated by the adapters TRIF and TRAM (Palsson-McDermott 2004).

Signaling mechanisms of NF-κB

One of the key factors for osteoclastogenesis, which is induced by osteoblasts, is RANKL, a member of the tumor necrosis family (Anderson 1997). RANKL was found to be expressed by T-cells (Wong 1997), and B-cells (Li 2000) as well as osteoblastic/stromal cells and is essential for osteoclast differentiation. RANK, which is one of the TNF receptor family members, is expressed in osteoclasts and their precursor cells as the receptor of RANKL (Josien 1999). Downstream signaling through RANK is essential for osteoclastogenesis. Deviation from the normal conditions of bone resorption results in bone disease such as periapical osteolysis and is thought to be caused mainly by infection with Gram-negative bacteria. Such an infectious and pathological condition induces inflammation, resulting in bone

resorption due to the modulation of physiological osteoclastogenesis, leading to a pathological bone resorptive condition (Hotokezka 2007).

There is a body of evidence that suggests that NF-κB plays an important role in gene regulation during inflammatory and immune reaction (Brand 1996) (Baeuerle 1996) (Barnes 1997). NF-κB regulatory sequences have been found in promoters or enhancers of numerous genes which encode for proinflammatory cytokines TNF and IL-1β, cytokines, several adhesion molecules and procoagulatory protein tissue factor (Siebenlist 1994) (Brand 1991) (Baldwin 1996). The typical NF-κB dimer consists of subunits p65 (Rel A) and p50, although other subunits have been identified (Siebenlist 1994). These complexes are present in an inactive state in the cytosol bound to inhibitory proteins, collectively termed IκB ((Siebenlist 1994). Several IκB proteins have been identified, including IκB-α (Thanos 1995), IκB-β (Thompson 1995) and IκBε (Whiteside 1997). A variety of agents, including factors such as LPS, TNF-α, and IL-1B, induce the activation of NF-κB (Müller 1993). A network of kinases mediates this activation and is accomplished by the phosphorylation of IκB, which is thus degraded in an ubiquitin-dependent step by the proteoasome, a multicatalytic high molecular weight protease system (Thanos 1995). The translocation of the activated NF-κB dimer into the nucleus is allowed by the removal of the inhibitor IκB.

The signaling mechanisms that lead to the phosphorylation of IκB, and thereby NF-κB activation, are only partly understood and characterized for TNF, Il-1β, and LPS (Stancovski 1997, Baeuerle 1998, May 1998, Kirschning 1998 and O'Connell 1998). An IκB kinase (IKK) complex, also named the signalsome, has been

identified as a true IκB kinase, with several of its active components being cloned, namely IKK-α, IKK-β, and NF-κB-inducing kinase (NIK) (DiDonato 1997). Two adaptor or scaffold proteins have also been found, IKK-γ/NF-κB essential modulator (NEMO)/IKK-associated protein 1 (Rothwarf 1998) and IKK complex-associated protein (IKAP) (Cohen 1998) which have been suggested to regulate the kinase activity. Additional kinases, such as mitogen-activated protein kinase/extracellular-regulated kinase kinase kinase-1 (Lee 1997) or mitogen-activated ribosomal S6 protein kinase (Schouten 1997) may assemble with the signalsome following activation of cells by certain stimuli. For example, activation of NF-κB by TNF appears to involve several upstream signaling proteins, which in turn activate NIK, thereby initiating a signaling pathway that results in IκB phosphorylation (Stancovski 1997).

LPS and RANKL Common Pathway

It has been suggested that IL-1 and RANKL promote the survival of osteoclasts through NF-κβ activation (Jimi 1998). The time course of changes in IκB's degradation in preosteoclast (pOC) cultures treated with LPS have been shown to be quite similar to that of RANKL (Suda 2002). Consistent, with the findings of a previous study (Jimi 1998), inhibition of NF-κB activation by lactacystin prevented the survival of pOCs supported by LPS or RANKL (Suda 2002). Lactacystin is a specific inhibitor of the 26S proteasome which is involved in the phosphorylation of the IκB's leading to the translocation of NFκB's into the nucleus to begin transcription. These findings suggest that LPS as well as RANKL support the survival of pOCs through NF-κβ activation (Suda 2002). Degradation of IκB's in pOC

cultures has been shown to be similar to that induced by adding LPS to purified osteoclast cultures (Suda 2002). These findings suggest that LPS directly acts on pOCs and elongates their life span through the activation of NF-κB (Suda 2002). Wortmannin– a specific P13k inhibitor – also blocked osteoclast formation; whereas, PD98059 – a specific MEK inhibitor – failed to affect RANKL- or LPS-induced osteoclast formation (Suda 2002) which is consistent with our results. Suda suggests that their results show that the effects of LPS and RANKL on pOCs must therefore be mediated not only by NF-κB, but by other signals such as P13/AkT. A lack of RANKL stimulation in response to LPS also showed that osteoclast formation during bacterial infection utilizes an alternative mechanism for activation (Coon 2007). In our study, in addition to the two pathway inhibitors used by Suda (2002), we also used SB202190 – a specific MAPK inhibitor, LY294002 – a specific P13K inhibitor, and FK506 – a Calcineurin/NFAT inhibitor. With the addition of these inhibitors, there were dramatic reductions in osteoclast formation for both the LPS and RANKL treated groups. As shown in the Suda study, using the same inhibitor of the MEK kinase pathway, there was no effect on osteoclast formation. Therefore, it can also be concluded that LPS and RANKL share a common pathway in osteoclast formation.

We also concluded that LPS-stimulated osteoclast formation is mediated through the TLR4 receptor and not RANK with the use of OPG and anti-TLR4 antibody. There is almost complete inhibition of osteoclast formation when cells treated with RANKL are cultured with OPG; whereas, there is no effect seen with the use of anti-TLR4. Conversely , when the LPS group was cultured with OPG, osteoclast formation occurred; however, when TLR4 was inhibited, there was a reduction in

osteoclast formation. The possible reasons for incomplete blockage of osteoclast formation in the LPS and Anti-TLR4 group is there may not have been a high enough concentration of antibody to block all the receptor sites. Secondly, LPS stimulates TNF-α secretion which has also been shown to induce osteoclast formation (Jiang 2006). Therefore, we propose that LPS may participate directly in osteoclast formation. We know that LPS directly stimulates osteoblasts to increase expression of RANKL. The RANK/RANKL interaction on osteoclast precursor cells can then sensitize these cells to LPS, which once bound to the TLR4 receptors, can act directly to induce osteoclast differentiation and activation through a shared pathway.

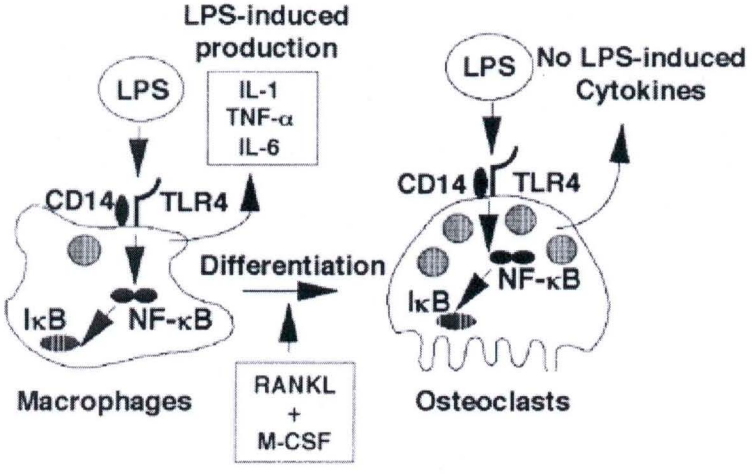
LPS effects on TNF-α release

LPS and TNF-α are two of the the most potent activators of NF-κB in cells of the monocytic lineage (Siebenlist 1994). However, the signaling cascades leading to NF-κB activation induced by these stimuli appear to differ markedly (Baeuerle 1998). Studies reveal that a completely different signalsome activation pattern results from incubation of cells with LPS and TNF-α (Fischer 1999). Incubation of human monocytic cells with LPS predominantly activated IKK-β with a peak of activation at 30 minutes and a second activation peak at 75 minutes which consisted of both IKK-β and IKK-α activity. This may be due to continuous stimulation by LPS in the medium and/or autocrine mechanisms. (Fischer 1999). TNF-α, in contrast, activated IKK-α preferentially, with a very early peak of activation at 5 minutes (Fischer 1999). The effect of TNF-α on IKK-β was 3-fold lower than that on IKK-α but showed a similar time course of activation with no second peak of activation (Fischer 1999). LPS appears to only modestly but preferentially stimulate IKK-β activity over

a longer time interval, whereas the effect of TNF-α on the IKK molecules is marked and rapid but appears to be rather limited (Fischer 1999). The effect of LPS on IKK activity is much weaker than that of TNF-α which may indicate that the IKK pathway represents just one of several parallel signaling pathways induced by LPS leading to NF- κB activation (Fischer 1999). It is also known that LPS is known to initiate signal transduction cascades resulting in the activation of a network of kinases, G-proteins, cyclic nucleotide metabolizing enzymes, lipid second messengers, and ion channels, some of which may not even be related to NF-κB (Sweet 1996). Additional kinases, however. may be involved in LPS-mediated IκB phosphorylation (Schouten 1997) or a modest but continuous activation of IKK by LPS may be as effective as the rapid but short-lived activation of IKK seen following TNF-α stimulation (Fischer 1999).

It is known that TNF-α, by activating NF-κβ, mediates LPS-stimulated osteoclastogenesis via its p55 receptor (Abu-Amer 1997). In our study, we examined the effects of LPS on TNF-α release during osteoclast formation with the use of ELISA. There was a differential response to LPS by monocytes and osteoclasts which has been shown in other studies (Itoh 2003). Osteoclasts showed a decreased response to LPS. These results suggest, that in terms of cytokine production in the presence of LPS, characteristics of monocytes and osteoclasts are quite different from each other which indicate that osteoclast precursors quit cytokine production (except for IL-6) in response to LPS as soon as the differentiation pathway of osteoclast precursors is determined. Loss of inflammatory responsiveness to LPS in osteoclasts must be requirement for performing essential roles in physiological bone turnover

(Itoh 2005). Further studies will be necessary to elucidate the mechanism of regulation of proinflammatory cytokine production in macrophages and osteoclasts.



Response to LPS	Macrophages	Osteoclasts
Survival	↑	↑
Activation of NF-κB	↑	↑
Cytokine production	↑	→

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TNF-α stimulation of Osteoclastogenesis

It is known that osteoclasts are derived from hematopoietic precursor cells of the monocyte-macrophage lineage and that osteoclast precursors produce proinflammatory cytokines such as TNF-alpha, IL-1Beta, and IL-6 in response to LPS. The way in which osteoblasts send a second signal to osteoclast precursors in response to primary osteolytic signals has been the subject of intense investigation. It is now known that osteoblasts regulate osteoclastogeneis by expressing RANKL (Yasuda 1998). Osteoclast precursors, which express the RANK, recognize RANKL

released by osteoblasts. In the presence of other costimulators such as M-CSF, RANKL stimulates the fusion of osteoclast precursors into multinucleated cells capable of resorbing bone (Parfitt 1998). Formation of osteoclasts is critical for normal development because it allows for the formation of the marrow spaces within bone and the eruption of teeth. Targeted deletion of RANKL in mice results in severe osteopetrosis and lack of osteoclasts, due to the inability of osteoblasts to support osteoclastogenesis (Kong 1999). Failure to produce M-CSF results in mice with osteopetrotic bone, and both types of genetic failure result in death of the animals after weaning because of malnutrition secondary to a failure of tooth eruption (Sundquist 1995). While RANKL has been considered to be a key regulator of osteoclastogenesis in mice, the paradigm is becoming complicated (Blair 2005). Many of the TNF-family receptors activate NF-κβ and are reported to replace or augment RANK signaling, particularly TNF-α receptors (Azuma 2000) (Lam 2000) (Komabayashi 2000) (Suda 2002) There are studies demonstrating that TNF-α induces formation of osteoclast-like cells independent of RANKL activity (Kobayashi 2000) (Azuma 2000).

TNF-α is a key component in the pathogenesis of inflammatory osteolysis; however, the way it recruits osteoclasts and promotes bone destruction are not completely known (Lam 2000). The indirect effect of TNF-α via osteoblast signals through regulation of RANKL and OPG is an accepted paradigm (Brandstrom 1998),and studies suggest that the RANK/RANKL system in mice and TNF-α expression are interdependent. TNF-α stimulates expression of the RANK receptor and RANKL, an action that leads to reciprocal stimulation of TNF-α in a positive

feedback manner (Hofbauer 1999, Komine 2001, Zhang 2001);however, controversy exists as to whether TNF-α can directly stimulate osteoclast differentiation. It has been shown that TNF-α, in the presence of M-CSF, is capable of inducing osteoclast differentiation (Kobayashi 2000) and that TNF-α alone can induce osteoclast formation and bone resorption (Azuma 2000). Mouse bone marrow cells that were cultured with M-CSF had formation of M-CSF bone marrow macrophages appear within 3 days. Exposure to RANKL or TNF-α stimulated the differentiation of these macrophages into osteoclasts in the presence of M-CSF. It has also been reported that when RANK (-/-) mice had osteotropic factors such as 1,25 $(OH)_2D_3$, PTHrP, and IL-1 administered, neither TRAP-positive cell formation nor hypercalcemia was induced (Li 2000). However, induction of TRAP+ cells occurred near the site of injection with TNF-α administration to RANK (-/-) mice, although the number of TRAP+ cells was not large. This suggests that TNF-α induces osteoclast differentiation in the absence of RANK-mediated signals in vivo (Katagiri 2002). However,consistent with our findings, Lam and associates found that a pure population of murine osteoclast precursors failed to undergo osteoclastogenesis when treated with TNF-α alone and that a small amount of RANKL strongly enhanced osteoclast formation suggesting that osteoclastogenesis induced by TNF-α occurred as a result of direct stimulation of macrophages exposed to a stromal environment that expressed permissive levels of RANKL (Lam 2000). Since their observations were in contrast to Kobayashi and Azuma, who proposed that TNF-α treatment of bone marrow macrophages induces them to acquire the osteoclast phenotype, Lam, et al. also used the same approach of three days of whole marrow culture prior to

macrophage isolation. Under those circumstances, they also observed that TNF-α directly induced osteoclast formation. However, when saturating levels of OPG were added at the initiation of whole bone marrow culture, rather than simultaneously with the addition of TNF-α after the initial three day culture period, TNF-α-stimulated osteoclastogenesis is completely absent (Lam 2000). The study of the mechanisms of osteoclastogenesis from osteoclasts generated in culture have been proven to be a vital tool. In the past, bone resorptive osteoclasts could only be differentiated reliably in culture by using primary bone marrow, as done by Azuma and Kobayashi. The marrow cultures contain osteoclast precursors and stromal cells/osteoblasts. Calcitoriol or parathyroid hormone, which are bone stimulatory agents, induce osteoblasts in the cultures to indirectly support osteoclast differentiation and activity by stimulating them to express RANKL (Roodman 1999). Determining whether exogenously added agents affect osteoclasts directly or indirectly is problematic in these heterogenous systems. In an in vitro culture system using osteoclast precursor cells purified from various tissues such as bone marrow, spleen, and liver, it is difficult to avoid contamination with other cell lineages such as T-cells and mesenchymal stromal cells. In this study, we used RAW264.7-a murine macrophage cell line that can differentiate into osteoclast-like cells in the presence of RANKL (Hsu 1999). There are some characteristic differences between RAW264.7 cells and macrophages; for example, RAW264.7 cells do not respond to IL-1β, and the osteoclast-like cells differentiated from RAW264.7 cells form smaller and shallower resorption pits on dentin slices than do osteoclasts derived from bone marrow

macrophages. However the cell line is useful to analyze the detailed mechanisms of osteoclast differentiation.

LPS dependence on TNF-α during osteoclastogenesis

Factors, such as TNF-α, IL-1, IL-6, parathyroid hormone, vitamin D_3, and prostaglandins. known to stimulate osteoclast formation, bind to receptors on osteoclast progenitors to induce the release of osteoclast-stimulating factors ((Boyce 1999). A genetically engineered soluble form of RANKL, in the presence of M-CSF, induced osteoclast differentiation of progenitors derived from mouse bone marrow cells, spleen cells, or human peripheral blood mononuclear cells in the absence of stromal cells/osteoblastic cells (Yasuda 1998). Typical osteopetrosis with complete occupation of the bone marrow space within endosteal bone is seen in RANKL-deficient mice, demonstrating that RANKL is essential for osteoclast development (Kong 1999). It has, however, been shown that multinucleation of preosteoclasts and/or commitment to the osteoclast lineage in the absence of stromal cells/osteoblastic cells, is induced by several inflammatory cytokines (Jimi 1999). c-src mRNA expression in purified bone marrow macrophages induced by LPS is a specific marker of commitment to the osteoclast lineage (Abu-Amer 1997). These findings suggest that osteoclast formation from progenitors can occur through the direct actions of several cytokines on osteoclast progenitors. Our results from investigating whether TNF-α can stimulate osteoclast formation directly, showed no osteoclast formation in cells treated with 50 ng/ml only of TNF-α or 50 ng/ml of RANKL for only 24 hours. However, with 10ng/ml and 50 ng/ml of TNF-α preceded by only 24 hours of RANKL, TRAP+ multinucleated cells were formed in the

culture. This indicates that TNF-α dose-dependently enhances in vitro osteoclastogenesis primed by a level of RANKL that is insufficient to induce osteoclast formation. This is in line with the Lam study. Furthermore, TNF-α stimulates osteoclastogenesis in circumstances both in vitro and in vivo in which osteoclast precursors, but not RANKL producing stromal cells are responsive to the cytokine (Lam 2000). Therefore, TNF-α targets both marrow stromal cells and osteoclast precursors, but directly impacts the latter only in the presence of permissive levels of RANKL. Discrepancies amongst studies can be attributed to the difference in the type of cell culture, culture conditions, concentrations uses, and the sensitivity in detection methods. It also reveals the intrinsic complexity in the regulatory mechanism.

Abu-Amer (1997) reported that LPS administered to wild-type mice promoted osteoclast formation. In contrast, LPS did not substantially enhance osteoclast formation in mice lacking the TNFR1, suggesting that LPS-induced osteoclastogenesis was mediated by TNF in vivo via this receptor. It was also reported that TNF-α stimulated the differentiation and survival of osteoclasts by a mechanism independent of the RANKL-RANK interaction (Kobayashi 2000). Other studies have shown that TNF-α stimulated multinucleated cell (MNC) formation in preosteoclast (pOC) cultures (Suda 2002). Such a stimulatory effect of TNF-α was observed in pOC cultures prepared from TNFR2 -/- mice but not TNFR1-/- mice. In contrast, LPS similarly induced osteoclast formation in pOC cultures prepared from both TNFR1-/- and TNFR2-/- mice. These findings suggest that signals mediated by TNFR1 rather than TNFR2 were involved in osteoclast formation in TNF-α treated

pOC cultures, and that LPS promoted the survival and fusion of pOCs independent of the TNF-α action (Suda 2002).

It has been previously reported that M-CSF stimulated osteoclast formation in pOC cultures (Takami 1999). In addition to this, anti-c-fms antibody which blocks the signal from M-CSF did not inhibit LPS-induced osteoclast formation in pOC cultures (Takami 1999). Thus, it is suggested that LPS stimulates osteoclast formation, at least in part, via a mechanism that is independent of such factors as RANKL, TNF, IL-1, and M-CSF, which are known to induce osteoclast formation in pOC cultures; therefore, not excluding the possibility that LPS-induced osteoclast formation is mediated by other unknown factors or pathways (Suda 2002). In our study, we pretreated cells with RANKL for 24 hours and it was removed. The cells were then treated with TNF-α, additional RANKL or LPS. These cells showed the expected osteoclast formation. However, the cultures of cells that were pretreated with the TNF-α antagonist, sTNF RI/Fc for 30 minutes showed abolishment or decreases in osteoclast formation. sTNF RI/Fc has been shown to be a potent TNF-α antagonist (Aggarwal 1996). It blocks TNFR1 which has been shown to enhance osteoclast formation; whereas, TNFR2 has been shown to inhibit osteoclastogenesis (Abu-Amer 2000). Osteoclast formation stimulated by TNF-α was blocked but osteoclast formation stimulated by RANKL was not affected. Osteoclast formation stimulated by LPS decreased 60% which shows that LPS-stimulated osteoclastogenesis is partially dependent on TNF-α indicating there must be other mechanisms involved in LPS-induced osteoclastogenesis which is consistent with the Suda study (Suda 2002). However, it must be kept in mind that TNFR1 and TNFR2-

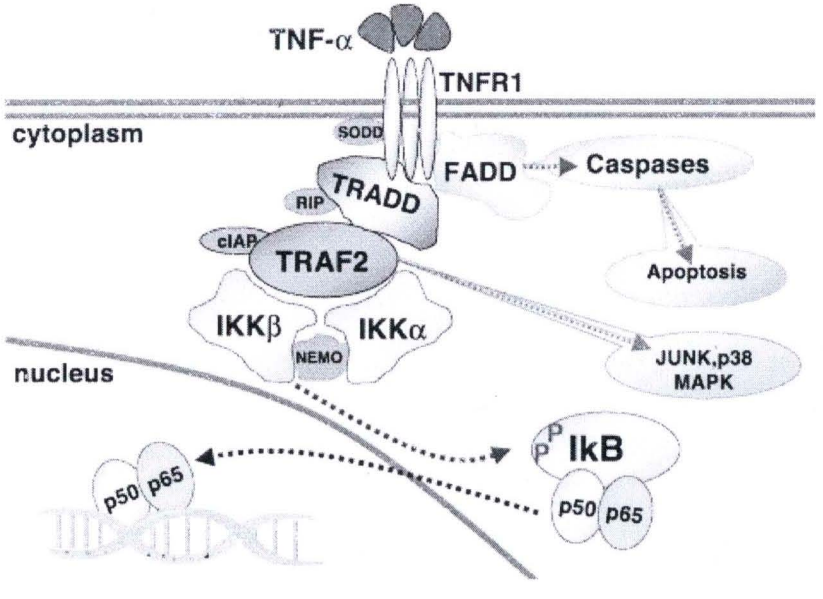
activated signaling pathways have been intensively investigated in a variety of other cell types and the current understanding is primarily based on the studies involving cells other than osteoclast precursors-osteoclasts. Since many of the pathways described in the literature, have been shown to be applicable to many different cell types (Locksley 2001); it is reasonable to assume that a considerable portion of the signaling cascades described may hold true for osteoclast precursors/osteoclasts (Feng 2005).

The LPS response system is highly controlled and complex and there are still major deficiencies in the understanding of the true nature of LPS signaling and how it might be controlled and regulated. It is still unclear as to how bacteria stimulate bone pathology and it is accepted that bacterial virulence factors invade into the periapical tissue and induce local pathology (Nair 1990). The connection between LPS and the development of periapical lesions and the accompanying bone loss is still controversial (Hong 2004). A positive correlation between LPS and the presence of periapical lesions has been reported (Sunqvist 1992). LPS levels in rat periapical lesions increased with time after lesion induction (Yamasaki 1992). Periapical bone destruction in dogs is induced with direct application of LPS to dental pulp (Mattison 1987). However, some authors argued about the significance of LPS in periapical lesion-induced bone loss in that periapical lesion extracts elicited obvious in vitro bone resorption, but LPS inhibitor could not alleviate this osteolytic activity (Stashenko 1992).

Future studies are also needed to evaluate whether these signaling cascades are indeed employed by oteoclast precursors/osteoclasts to mediate TNF-α dependent

activation of the NF-κB, JNK, p38, ERK and AkT pathways. Additionally, further studies will help clarify the intracellular signaling pathways from LPS in preosteoclasts and osteoclasts, which will contribute to the understanding of the pathogenesis of inflammation induced bone resorption.

In conclusion, what this data shows is that LPS and RANKL share a common pathway in the activation of osteoclastogenesis. There is a differential response by monocytes and pre-osteoclasts during LPS-stimulated TNF-α release. LPS-stimulated osteoclastogenesis is partially dependent on TNF-α; therefore, there must be other mechanisms involved independent of this pathway. Lastly, TNF-α cannot directly induce osteoclastogenesis unless primed by RANKL for at least twenty-four hours.



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Investigations of Mechanisms Involved in LPS-Stimulated Osteoclastogenesis

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A Thesis

Submitted in Partial Fulfillment of the

Requirement for the Degree of

Master of Dental Science

at the

University of Connecticut

2007